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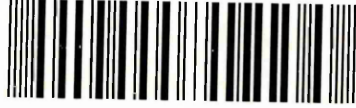
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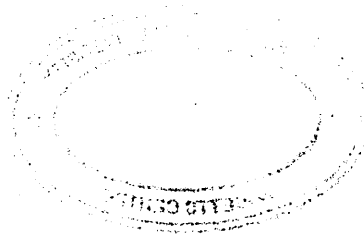
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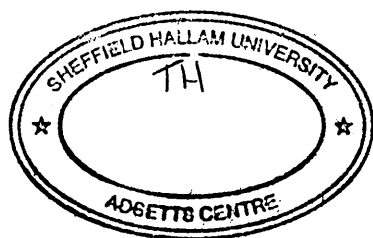
Ana B. Cáceres

**A thesis Submitted in partial fulfilment of the
requirements of Sheffield Hallam University**

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ABSTRACT

Methods for both the on-line and off-line fluorimetric determination of total selenium and selenium species using 2-(α -pyridyl)-thioquinaldinamide (PTQA) as a fluorimetric reagent have been developed. As PTQA is not available on the market, methods for its synthesis were critically reviewed, and in the light of the information obtained, modifications were made to the synthesis and purification conditions in order to improve yield from 16 % to 38.9 %. The synthesised PTQA was used for the optimisation of a batch fluorimetric method based on the reaction between the reagent and Se (IV) in acidic medium. The developed method was applied to the determination of total selenium in nutritional supplements after microwave digestion and photoreduction of Se (VI) to Se (IV). The analytical precision in the concentration range 0.5 – 1000 $\mu\text{g mL}^{-1}$ was better than 5 %. Excellent agreement ($r^2 = 0.9960$) between the results of the fluorimetric and ICP-MS methods was obtained when a reference material, TMDA 51.2 supplied by National Water Research Institute Environment Canada and three nutritional supplements were analysed.

A novel automated fluorimetric method for the simultaneous determination of Se (VI) and Se (IV) is described in which a purpose built flow injection system was used for the on-line photoreduction of Se (VI) to Se (IV) before detection. Variables such as tube diameter, flow rates, type of acids (HNO_3 , H_2SO_4 , CH_3COOH , H_3PO_4 and HCl) that affect the rate at which PTQA reacted with Se (IV) were investigated and optimised. The linear calibration range was found to depend on the type and concentration of acid. The most intense fluorescence signals were observed in HNO_3 and at a PTQA concentration of 5×10^{-4} M. A sample throughput of 8 samples/hr was achieved. Method validation was by comparing the results obtained for the determination of selenium in nutritional supplements with those from ICP-MS.

Selenium speciation in the nutritional supplements was studied using a developed method in which the selenium species selenomethionine, Se (IV), and Se (VI) were extracted, separated by HPLC prior to on-line photoreduction of Se (VI), and post column hydride generation before ICP-MS detection. Separation of the three species was achieved with excellent resolution in less than 18 minutes. The limit of determination for the three species was 0.50 $\mu\text{g L}^{-1}$.

Dedicated to all people that believes in dream and works for them,
to Dr. Humberto Nevado R. because you believe in academy.

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Selenium, discovered by Berzelius in 1817, is widely distributed in the earth's crust with an abundance of 7×10^{-5} weight percent. Selenium is sandwiched between oxygen, sulphur, tellurium and polonium in group VIA of the periodic table. The chemical and biochemical behaviour of selenium closely resembles those of sulphur and tellurium. Indeed, all compounds of the element have sulphur analogues. A review of the chemical and biochemical properties of selenium is given below.

1.1 PROPERTIES OF SELENIUM

1.1.1 Chemical of selenium

The outer electronic configuration of selenium is $4s^2 4p^4$, enabling it to form divalent compounds with two covalent bonds and two lone pairs (1). The commonly encountered oxidation states of selenium are -2, 0, +4, +6. The inorganic compounds of selenium are, generally, similar to those of sulphur. Selenium burns in air with a blue flame to produce the following oxides such as SeO , SeO_2 , and Se_2O_3 . The commonest and the most stable oxidised form is SeO_2 , which adsorbs water to produce selenous acid (H_2SeO_3), a weak oxidising dibasic acid. Selenous acid is a stronger oxidising agent than sulphurous acid as judged by its quantitative oxidation of iodide in acidic solutions. Selenous acid is readily oxidised by halogens in the presence of silver ions or 30 % H_2O_2 to produce selenic acid, H_2SeO_4 . Selenic acid is as strong but less stable than sulphuric acid, and it is more easily reduced by hydrobromic and hydroiodic acid to form selenious acid or elemental selenium. Selenium reacts with hydrogen and halogens, except iodine, to form selenides and halides. Se_2Br_2 is more stable than S_2Br_2 . Similarly, SeCl_4 is more stable than SCl_4 but less stable than TeCl_4 , (2, 3).

Selenium dissolves in strongly alkaline solution to produce selenides and selenites. Elemental selenium reacts with alkali-metal cyanides to yield selenocyanates, MSeCN . Reaction with alkali-metal sulphites yields selenosulphates, MSeSO_3 .

The only compound of selenium with hydrogen is hydrogen selenide; H_2Se is obtained by heating selenium in hydrogen



Hydrogen selenide is a colourless flammable gas with an offensive odour and greater toxicity and lower thermal stability than hydrogen sulphide. Its solubility in

water is greater than that of hydrogen sulphide and is a stronger acid than hydrogen sulphide (2).



1.1.2 Biochemical properties

Selenium was recognised as an essential trace element by Schwarz, but it was not until the early 1970's that it was shown to be an essential component of the enzyme glutathione peroxidase (GPX or GSH-Px) (4). This enzyme is involved in the protection of cells against oxidative damage caused by reducing hydroperoxides; a role shared with vitamin E. Several Se-containing enzymes are now recognised (5) including five GSH-Px and three iodothyronine. More recently, Combs (6) and Rayman (4) have added eight more to the list. Table 1. 1 shows known selenium proteins.

Recently, the pathways of selenium uptake have been proposed by Combs as shown in Figure 1.1. The inorganic selenium forms (selenate, selenite) are reduced to yield hydrogen selenide (H_2Se), which is incorporated into selenoproteins. Selenomethionine (SeMet) the common form of selenium in foods is incorporated non-specifically into proteins in place of methionine. Selenocysteine (SeCys) which is a product of SeMet catabolism is metabolised to H_2Se . CH_3SeH is formed from methylselenocysteine (CH_3SeCys) present in some foods (Allium vegetables). Excess H_2Se not used for the synthesis of selenoproteins is methylated to form dimethylselenide ($(\text{CH}_3)_2\text{Se}$) and trimethylselenonium ($(\text{CH}_3)_3\text{Se}^+$) and excreted in the breath and urine, respectively. The oxidation of H_2Se excess leads to production of superoxide, as SeO_2 , and other reactive oxygen species such as H_2O_2 .

**Table 1. 1 Known selenoproteins, which carry out nutritional functions of selenium,
from Rayman, M. (4).**

Selenoprotein	Function
Glutathione peroxidases (GPx1, GPx2, GPx3, GPx4)	Antioxidant enzymes: remove hydrogen peroxide, and lipid and phospholipid hydroperoxides (thereby maintaining membrane integrity, modulating eicosanoid synthesis, modifying inflammation and likelihood of propagation of further oxidative damage to biomolecules such as lipids, lipoproteins and DNA).
(Sperm) mitochondrial capsule selenoprotein	Form of glutathione peroxidase (GPx4): shields developing sperm cells from oxidative damage and later, polymerises into structural protein required for stability/mobility of mature sperm.
Iodothyronine deiodinases (three isoforms)	Production and regulation of level of active thyroid hormone, T3, from thyroxine, T4.
Thioredoxin reductases (probably three isoforms)	Reduction of nucleotides in DNA synthesis: regeneration of antioxidant systems; maintenance of intracellular redox state, critical for cell viability and proliferation of gene expression by redox control of binding of transcription factors to DNA.
Selenophosphatase, SPS2	Required for biosynthesis of selenophosphate, the precursor of selenocysteine, and therefore for selenoprotein synthesis.
Selenoprotein P	Found in plasma and associated with endothelial cells. Appears to protect endothelial cells against damage from peroxynitrite.
Selenoprotein W	Needed for muscle function.
Prostate epithelial selenoprotein (15KDa)	Found in epithelial cells of ventral prostate. Seems to have redox function (resembles GPx4), perhaps protecting secretory cells against development of carcinoma.
DNA-bound spermatid selenoprotein (34KDa)	Glutathione peroxidase-like activity. Found in stomach and in nuclei of spermatozoa, May protect developing sperm.
18 KDa selenoprotein	Important selenoprotein found in kidney and large number of other tissues. Preserved in selenium deficiency.

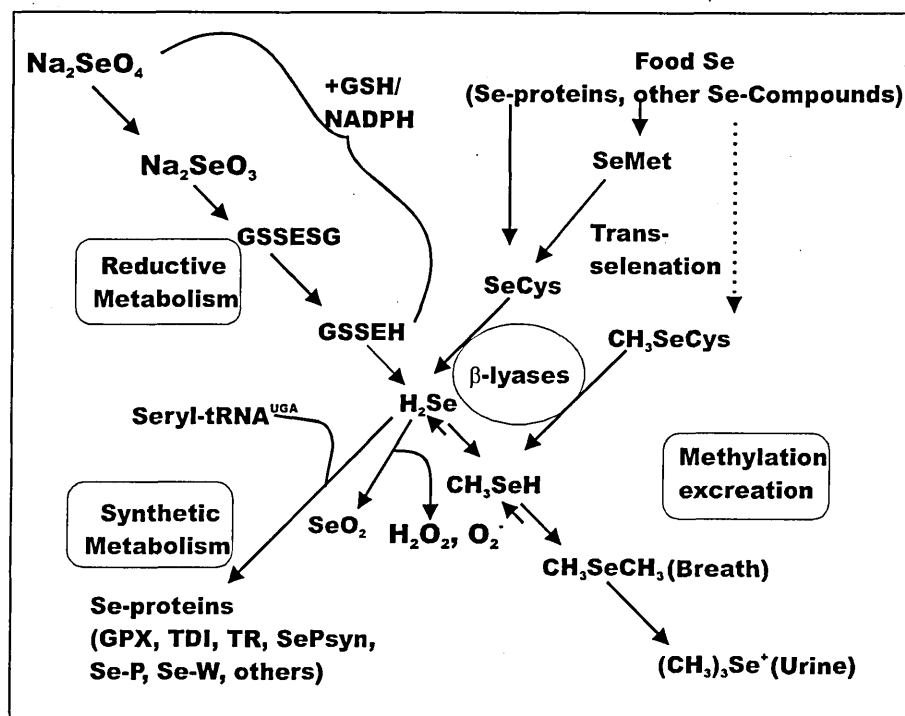


Figure 1.1 Pathways of Se metabolism (6).

1.2 SELENIUM DEFICIENCY IN HUMANS

Chronic selenium deficiency known to occur in areas with low selenium in soils in China has been linked to the development of Keshan disease, juvenile cardiomyopathy, and chondrodystrophy Kaschin-Beck disease, a condition affecting the joints and bones. Both of these diseases are endemic in parts of China and Russia. In China very low as well as very high concentrations of selenium have been found. Studies by Tan et al. (7) reported lowest values of 0.022 mg Kg^{-1} and highest of 3.806 mg Kg^{-1} . In the 1940's, the levels of fatalities was about 80% in areas with low soil selenium. There has however been a decline in fatalities in recent years to less than 30 % apparently as a result of better medical care. Dramatic reductions in the incidence of the Keshan disease have been achieved by the prophylactic administration of oral tablets containing Na_2SeO_3 (0.5-1 mg Se/child per week) or selenite-fortified table salt 10-15 mg Se/Kg, (6). These improvements in the health of the population in affected areas have demonstrated the importance of an adequate selenium intake. A number of epidemiological studies have linked selenium deficiency with an increased susceptibility to certain types of cancer (5, 6), vascular disorders including coronary heart disease, arteriosclerosis and platelet hyperaggregability, anaemia and hypertension; rheumatoid arthritis, multiple sclerosis, muscular dystrophy, cystic fibrosis, asthmatic inflammation, HIV/AIDS, skin conditions as psoriasis and abnormalities in the immune system, (5, 6). Low plasma Se levels have been found to be associated with increased risk of

respiratory morbidity among low-birth-weight newborns. Low Se levels have also been associated, pre-eclampsia, spontaneous abortions, and male infertility. Studies on patients with several other diseases have shown low concentration of the element. However, it is important to point out that in most of these conditions it is unclear whether the low selenium levels is as a result of the disease or due to low selenium intake (6).

1.3 EFFECTS OF ADEQUATE SELENIUM INTAKE

Several studies have found evidence of the anti-carcinogenic potential of Se. An inverse relationship between the intake of Se and cancer risk has been shown. Studies in rat, mince and pig have shown that intake of Se can inhibit tumourigenesis (6). A Nutritional Prevention of Cancer (NPC) trial carried out in USA showed that selenium supplementation at 200 µg Se/d (in the form of Se-enriched yeast) was associated with significantly lower incidences of total non-skin cancer (37 %), total carcinomas (45 %), with reductions of 63%, 58%, and 46% in the incidences of prostate, colon-rectum and lung cancers, respectively (6). In addition, mortality due to lung (53%) and total cancers (50%) were reduced. These findings demonstrate the importance of selenium supplementation in human health and disease.

The main source of selenium is the diet but adequate intake of the element is assured only when there are adequate levels in the soil. Recently there has been an increase in the use of selenium-containing diet supplementation in a bid to meet the recommended intake of the element. The form of Se in those supplements are either, organic, selenomethionine, or inorganic, selenite or selenate or a mixture of both. However there is still no conclusive evidence to suggest which selenium form is more bioavailable inspite of the number of studies (4 -8) that have been carried out. A study by W. Windisch et al (9) concluded that the bioavailability of the element is as follows: Se (IV) > selenocysteine >>> selenomethionine. They found that selenium amino acids can be absorbed to a higher extent than selenite. However, selenium amino acids are less available for metabolism.

1.4 SELENIUM TOXICITY

There is a narrow range between the concentrations at which selenium is essential and toxic. The toxic effects of the element were first observed in animals grazing in

seleniferous areas (5, 6, 10). In parts of China where soil and coal selenium were high (8 and 84 g Se Kg⁻¹), respectively, morbidity was about 50%. The symptoms include loss of hair and nails, as well as skin lesions, such as erythema, oedema, eruptions, intense itching; in addition, hepatomegaly, polyneuritis and gastrointestinal disturbances were observed. Intoxication is thus always due to compounds such as selenites, selenates, and organic selenium compounds found in plants. An oral dose 10 mg Kg⁻¹ of selenite per body weight is lethal for pigs, dogs, and cats, whereas 4 mg Kg⁻¹ of metallic selenium is tolerated without adverse effects. An oral intake of 5 to 15 mg selenate per Kg of body weight results in chronic intoxication. Ingestion of plants containing 45 mg Kg⁻¹ organic bound selenium is fatal to sheep but the same amount of selenium taken as selenite does not cause poisoning. A daily dose of 1 or 0.5 mg Kg⁻¹ body weight, respectively, causes poisoning after a few days in cattle and sheep. Acute poisoning characterised by irritation, gastroenteritis, vomiting, diarrhoea, and collapse ("blind staggering") appears after ingesting feed with a selenium content of more than 20 mg Kg⁻¹ for four weeks. Other symptoms include loss of appetite, central nervous paralysis, ataxia, emaciation, psycho-depression, and myopathy in lambs and calves. In horses, hair loss in the mane and tail are observed, and cattle lose the tips of their tails. Detachment of the hooves occurs in horses. Selenium is teratogenic in sheep. The main symptom of chronic poisoning, known as alkali disease, is emaciation, which appears when feed contains more than 10 mg Kg⁻¹ of selenium. Selenium intoxication can be reduced by using protein-rich feed (11).

Hydrogen selenide is the most acutely toxic selenium compound. Exposure to hydrogen selenide by inhalation results in primary respiratory effects, such as irritation of the mucous membranes, pulmonary oedema, severe bronchitis, and bronchial pneumonia. The only selenium compound that has been shown to be carcinogenic in animals is selenium sulphide, which increased the incidence of liver tumours in rats and mice after oral exposure. Selenium sulphide is a pharmaceutical compound used in anti-dandruff shampoos and has different properties compared to the inorganic selenium compounds found in foods and the environment (12). Combs (6) has reviewed the selenium levels in blood around the world, and the results can be classified into three groups as shown in Table 1. 2 Se concentrations in the range of 72 – 120 µg L⁻¹ are taken as normal values.

Table 1. 2 Global distribution of Se level in blood based on data reported by Combs et al (6)

Selenium level	Country		
Low (< 75 mg L ⁻¹)	Austria	Czech Republic	Northern Ireland
	Bulgaria	Estonia	Poland
	Burundi	Finland before 1984	Russia, Trans-Baikal
	China, non-Keshan disease areas	Greece	Bosnia Herzegovina
		Hungary	
	China, Keshan disease areas	New Zealand, south island	Croatia
			Serbia
	Cuba	Nigeria	Yugoslavia
			Zambia
Normal (75 – 120 mg L ⁻¹)	Australia	Republic of Ireland	
	Azerbaijan	Israel	Scotland
	Belgium	Italy	Singapore
	Canada	Jamaica	South Africa
	China, eastern urban areas	Mexico	Spain
		The Netherlands	Sweden
	Colombia	New Zealand, north island	Switzerland
	Denmark		Taiwan
	Egypt	Niger	Turkey
	England	Norway	USA, eastern and southern states
	Finland after 1984	Portugal	
	France	Russia, eastern, Ural, and Siberia	Hawaii
	Germany		Uzbekistan
	India	Saudi Arabia	Zaire
	Chile	Greenland	USA, central and western states
	China, Kaschin-Beck disease area	Guatemala	
		Japan	Venezuela
	Georgia	Libya	Vietnam

1.5 SOURCES OF SELENIUM

1.5.1 Soils and rock

Major contributions to the global distribution of selenium include: continental and volcanic dusts, industrial particulates and volcanic gas, and the burning of fossil fuel, each of which contribute 3×10^8 , 1×10^8 , 50×10^8 , 0.3×10^8 , and 90×10^8 g year⁻¹, respectively, of the element. Soil Se content ranges from 0.02 to 2.5 $\mu\text{g g}^{-1}$ (2). Sedimentary rocks are the major materials which make up agricultural soils. The concentration of the element in sedimentary rock and sandstone ranges between 0.08 to 1 $\mu\text{g g}^{-1}$ and 0.05 to 1 $\mu\text{g g}^{-1}$, respectively. Carbonate rocks contain between 0 to 20 $\mu\text{g g}^{-1}$ of the element. The concentration of Se in coal ranges from 0.1 to 4 $\mu\text{g g}^{-1}$. Consequently, when coal or oil are burned, Se is introduced into the atmosphere and deposited far away from the source by precipitation.

Soil Se content ranges from 0.02 to 2.5 $\mu\text{g g}^{-1}$. In acid ferruginous soils, selenium is found as a basic selenite or strongly adsorbed to iron (III) oxide (6, 2).

1.5.2 Selenium in waters

In natural waters, Se can occur in four oxidation states: selenate (VI), selenite (IV), elemental selenium (0), and selenide (-II), in a variety of organic and inorganic compounds. Chemical equilibrium is rarely achieved with respect to the distribution of the various oxidation states. Selenate species should thermodynamically predominate in well-oxygenated surface waters, but this is frequently not the case. Most of the transformations of Se appear to be microbially mediated and its methylation is of both biological and environmental significance (1, 3). Surface, ocean and well waters contain less than 0.05 mg L⁻¹ selenium, but spring and irrigation drainage waters contain over 1 mg L⁻¹. The average concentration of selenium in the major oceans is 0.09 mg L⁻¹. Higher concentrations of Se have been observed in acidic and weakly basic waters of pH 2.4 – 3.0 and 7.4 – 8.0, respectively. In the United States, levels in drinking water exceeding 0.01 mg mL⁻¹ are classified as potentially dangerous and could constitute grounds for rejection of the water supply (2, 3).

1. 5.3 Selenium in plants

The normal selenium level in plants is 0.01 to 0.1 mg Kg. Soil concentrations of 100 mg Kg⁻¹ result in plant levels of up to 50 or even 100 mg Kg⁻¹ (11). The Se content in plants depends on a number of factors such as the level of the element in the soil, type of plant, and the soil conditions. Indicator plants which take up selenium can be divided into two categories: primary and secondary. Primary indicators include plants such as Astragalus, Machaeranthera, Haplopappus and Stanley for which Se is essential for their growth, and contain several mg g⁻¹ of the element. Secondary indicators do not require selenium for their growth; these include Aster, Atriplex, and Grndelina, which contain the element when they are grown in selenium-containing soils. Se is taken up by plants either as selenate, selenite or organic selenium. Selenate and sulphate ions are taken by similar processes in the plant root. Selenite ions are more easily absorbed and assimilated than selenate.

The uptake of Se in plants can be inhibited by sulphur because their compounds compete for similar transport pathways across cell membranes. This is particularly evident in the antagonism between sulphate and selenate, or between methionine and selenomethionine.

A list of the selenium compounds found in plants is shown in Table 1. 3.

Table 1. 3 Selenium species in plants (13).

Compound	Formula	Source
Selenocystathionine	$(\text{HOOC-CHNH}_2\text{-CH}_2\text{)}_2\text{Se}$	Selenium accumulating plants
Selenocysteine	$\text{HSe-CH}_2\text{-CHNH}_2\text{-COOH}$	Non-selenium accumulating plants
Selenocystine	$(\text{HOOC-CHNH}_2\text{-CH}_2\text{)-Se-Se-CH}_2\text{-CHNH}_2\text{-COOH}$	Selenium accumulating plants
Selenohomocystine	$\text{HOOC-CHNH}_2\text{-CH}_2\text{-CH}_2\text{-Se-Se-CH}_2\text{-CHNH}_2\text{-COOH}$	Selenium accumulating plants
Methyl selenocysteine	$\text{CH}_3\text{-Se-CH}_2\text{-CHNH}_2\text{-COOH}$	Non-selenium accumulating plants
Selenomethionine	$\text{CH}_3\text{-Se-CH}_2\text{-CH}_2\text{-CHNH}_2\text{-COOH}$	Non-selenium accumulating plants

The Se compounds supplied to the plant may also influence the proportions of accumulated organic selenium and selenate in the stem and leaves; Astragalus shows a selenium distribution where the organoselenium compounds are accumulated only in the stalk and leaves while selenite is found mainly in the roots. In addition, when the concentration of selenate in soils is decreased, the proportion of the organic forms is increased (2).

1.5.4 Selenium in food

The minimum selenium requirement in food is 0.04 mg Kg^{-1} . A level of about 0.1 mg Kg^{-1} is optimal and concentrations exceeding 4 mg Kg^{-1} may lead to toxic symptoms (11). Most fruits and vegetables generally contain less than $0.01 \text{ } \mu\text{g g}^{-1}$ Se, except for garlic, mushrooms and radish, which contain 0.25, 0.13 and 0.04 mg g^{-1} , respectively. Wholewheat flour and bread contain two to four times more selenium than white flour and bread. The Se content of egg white is about three times less than the yolk; brown sugar contains about four times more Se than white sugar. The quantity of Se in meat samples varies over a wide range. In red meat, the selenium concentrations range between 0.01-0.03 or 0.27-0.91 $\mu\text{g g}^{-1}$ as shown in Table 1. 4. The selenium concentrations depend upon a number of factors such as the anatomical location of the meat. The distribution in animals shows that vital organs such as the liver and kidney usually contain the highest selenium levels, although high levels can also be found in the pancreas, spleen, heart or lungs (6).

Table 1. 4 Typical selenium contents in food ($\mu\text{g g}^{-1}$) (6).

	Food			Class				
	Cereal	Vegetables	Fruits	Red meats	Poultry	Fish	Milk	Eggs
USA	0.06-0.66	0.001-0.14	0.005-0.06	0.08-0.50	0.01-0.26	0.13-1.48	0.01-0.26	0.06-0.20
England	0.02-0.53	0.01-0.09	0.005-0.01	0.05-0.14	0.05-0.15	0.10-0.61	0.01-0.08	0.05-0.2
Germany	0.03-0.88	0.04-0.10	0.002-0.04	0.13-0.28	0.05-0.15	0.24-0.53	0.01-0.10	0.05-0.20
Finland 1	0.005-0.12	0.001-0.02	0.002-0.03	0.05-0.10	0.05-0.10	0.18-0.98	0.01-0.09	0.05-0.20
Finland 2	0.01-0.27	0.01-0.02	-	0.27-0.91	-	-	0.01-0.25	0.02-0.15
New Zealand	0.004-0.09	0.001-0.02	0.001-0.004	0.01-0.04	0.05-0.10	0.03-0.31	0.003-0.025	0.24-0.98
China 1	0.005-0.02	0.002-0.02	0.001-0.003	0.01-0.03	0.02-0.06	0.03-0.20	0.002-0.01	0.02-0.06
China 2	0.017-0.11	0.002-0.09	0.005-0.04	0.05-0.25	0.05-0.10	0.10-0.60	0.01-0-03	0.05-0.15
China 3	1.06-6.9	0.34-45.7	-	-	-	-	-	-
Venezuela	0.123-0.51	0.002-2.98	0.005-0.06	0.17-0.83	0.10-0.70	0.32-0.93	0.11-0.43	0.50-1.5

Finland 1: before 1984 and 2: after 1984. China 1: non-Keshan disease areas, 2: Keshan disease areas, and 3: China, Kaschin-Beck disease area, selenosis area.

1.6 SELENIUM USES

The photoelectric and semi-conducting properties of selenium are exploited in photocell devices, solar batteries, speciality transformers, rectifiers and photocopiers. Selenium compounds are used in pigments and in colour glass manufacture where they are used for decorative effect and to limit heat and glare transfer. In addition they are used as catalysts in petroleum processes and in organic synthesis. In metallurgy, selenium is used as an additive in unalloyed and alloyed steels to the machining properties of steel, photocopy machines, rubbers, ceramics etc. (2,3)

1.7 METHODS OF ANALYSIS

The determination of the total Se in biological materials is now a routine task for many laboratories; however, a few problems still remain. A review of the various methods of selenium pretreatment and determination are given below.

1.7.1 Separation and preconcentration

1.7.1.1 Volatilisation

Hydrogen selenide is formed when Se (IV) is reduced with NaBH_4 in a strong acid medium. Selenium (VI) is reduced to Se (IV) before hydride generation, usually with boiling 4-7 M HCl. To avoid the back-oxidation of Se (IV) by residual Cl_2 , the system must be flushed with an inert gas during the process. It is advisable to strip the hydride from the system at low pH in order to avoid dissociation. The efficiency of the Se (IV) reduction with NaBH_4 is increased if iodine or bromide is present during the reduction step. The reaction is accelerated by using a HBr medium for prereduction and hydride generation. Generation of H_2Se is interfered by the presence of metals forming fine precipitates of which are formed upon reduction with NaBH_4 and which can capture and decompose the H_2Se . Other interferents include hydride-forming elements, which compete for the reagent, and redox species. These types of interference have been comprehensively discussed (14). The mechanisms have been studied with emphasis on bivalent metals (Sn (II), Ni, Co, Cd, Zn, Fe (II)) and Cu, Ag and Au. Interferences can be alleviated by increasing the HCl and decreasing the NaBH_4 concentrations, masking with 1-10-phenanthroline or hexacyanoferrate (III) or addition of Fe (III) that acts as a redox buffer. The most troublesome is the interference with Cu. Separation or masking copper can remove this type of interference. Ni is also an interferent that can be masked with citric acid or removed. Lead can be precipitated as PbCl_2 . The sources of systematic errors in HG-AAS have been studied by the radiotracer technique (14).

Various semiautomated or automated systems for hydride generation have been developed (14). Experimental detection limits can be enhanced by trapping the H_2Se on a Pd-modified graphite furnace or cryogenically on GC packing. The memory effects related to the deposition of unreacted analyte, metal selenide (e.g., CuSe), or H_2Se on the surface of the Teflon mixing tube and gas-liquid separator have been discussed. Other volatilisation methods include the distillation of SeBr_4 or SeCl_4 from concentrated HBr and HCl, respectively, or the formation of volatile species by reacting Se (IV) with NaBEt_4 or 1,2-diaminobenzene and its derivatives.

1.7.1.2 Extraction

Selenium (IV) has been extracted as SeI_4 into toluene. Piazselenols formed by the reaction of Se (IV) with 1,2-diaminobenzene or 2,3-diaminonaphthalene or its derivatives are readily extractable into nonpolar solvents. Extraction of Se (IV) with dithiocarbamates while interferents are masked with ascorbic acid is an alternative procedure (14).

1.7.1.3 Sorption

Sorption of Se on thiol cotton fibre, Bismuthiol II sulphonate and anion-exchange resins have been reported. Sorption of the Se-APDC complex on activated carbon is an alternative (14).

1.7.2 Titrimetric methods

Titrimetric methods have been used for the determination of milligram amounts of Se. This method is based on the formation of sparingly soluble selenium compounds, and can be classified into four groups: as reduction of Se (VI) to Se (IV), and of Se (IV) to Se (0) and oxidation of Se (IV) to Se (VI) and Se (0) to Se (IV) or Se (VI). In the iodometric determination, an excess of potassium iodide is added and liberated iodine is titrated with standard sodium thiosulphate solution. The disadvantage of the method is that the starch-iodine end point is rendered less sharp by red selenium. It can be prevented by extracting the liberated iodine with an organic solvent, such as chloroform or carbon tetrachloride. Similarly, Te (IV) forms the complex, TeI_6^{2-} , which quantitatively reacts with thiosulphate $\text{Na}_2\text{S}_2\text{O}_3$. To prevent the hydrolysis of $\text{Te}(\text{S}_2\text{O}_3)_2^{2-}$, an excess of sodium thiosulphate is necessary (2).

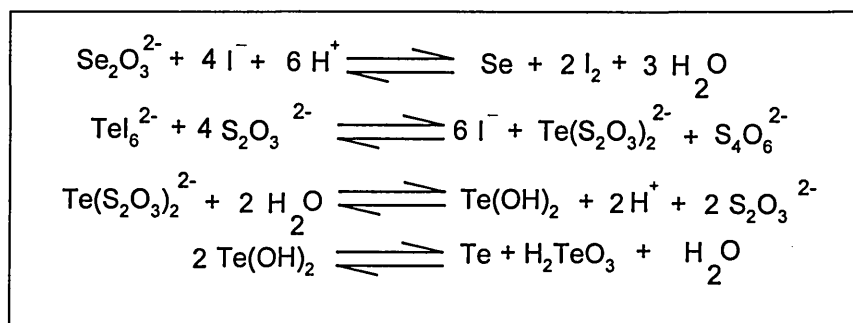


Figure 1. 2 Reaction for the iodometric determination of selenium (2).

1.7.3 UV-visible spectrophotometric methods

Spectrophotometric methods are more sensitive and selective than both gravimetric and titrimetric methods. Spectrophotometric measurements are based on the formation of complex compounds. For the colorimetric determination of selenium, reductants such as tin (II) chloride or hydrazine can be used. The solutions of the elements obtained by such reduction absorb at 390 nm and 259-260 nm, respectively. Gum arabic or poly(vinyl alcohol) can be used as solution stabilisers.

Methods based on the formation of piaszelenols with O-diamines, complexes with organic reagents containing sulphur, diazonium with organic reagents containing sulphur, diazonium salts by oxidation of organic compounds, and Se (II) complexes with semicarbazide and thiocarbazide are known. Reaction of Se (IV) with 3,3'-diaminobenzidine (DAB) at pH 2.5 gives an intense yellow compound, diphenylpiaszelenol, that is extracted at pH 5 with toluene. EDTA is used to mask the interference from doubly charged cations. The most widely used is the method based on the reaction of Se (IV) with 3,3'-diaminobenzidine to form the yellow piaszelenol either in the aqueous phase or on extraction into toluene ($\epsilon = 1 \times 10^4$) at 420 nm (2, 14).

1.7.4. Spectrofluorimetric methods

Spectrofluorimetric methods are usually not sufficiently sensitive for biological specimens; however, toxic concentrations of the element can be determined. Monopiaszelenol is formed from diaminobenzidine (DAB) and selenious acid. Se in the sample should first be converted to Se (IV), which forms a monopiaszelenol, extractable in an organic solvent. The reaction rate of 2,3- diaminonaphthalene (DAN) produces a complex similar to that produced by DAB but with a higher intensity signal. Another fluorescent method used recently is based on the oxidation of 2- (α -Pyridyl) thioquinaldinamide (PTQA) by Se (IV) in acidic medium. This method does not require extraction before the fluorescence measurement, and detection limits of $10 \mu\text{g L}^{-1}$ have been reported (14, 15).

1.7.5 Atomic Absorption Spectrometry (AAS) methods

Flame AAS offers a sensitivity of $0.6 \mu\text{g mL}^{-1}$ in the recommended air-C₂H₂ oxidising (lean) flame when measurements are carried out at 196.1 nm. Because of

considerable radiation scatter, background correction is essential in order to improve accuracy.

Methods based on AAS have been used extensively for the determination of Se. These include hydride-generation AAS in which hydrogen selenide is formed as a volatile analyte by the reduction of Se with reagents such as NaBH_4 (see section 1.7.1.1 and 1.8.3.3), Zn, KI, and SnCl_2 . Noble metals, oxidants, potassium iodide and some ions can interfere with the analysis. To separate the interferents, selenium is extracted from the aqueous solutions using an appropriate organic compound, leading to increased sensitivity (14).

1.7.6 Graphite furnace atomic absorption spectrometry methods

Graphite furnace AAS offers a detection limit of 10 pg. Iron oxide formed in the presence of oxygen is the major interferent causing overcorrection when D_2 background correction is used. The remedies include either Zeeman background correction or the addition of CO or Pt. Other spectral interferences are caused by the presence of Ni and P_2 . Deuterium correction may not compensate for the background of Ca and Mg phosphates, so for routine analysis, Zeeman correction should be used. Volatility losses can also cause errors in this determination. Selenium, if present as organoselenium, can be lost during the ashing and atomisation stages because of the formation of volatile H_2Se or SeO_2 . The thermal behaviour is dependent on the oxidation states (14). Nickel stabilises Se (IV) and Se (VI) in the presence of NaCl and Na_2SO_4 and also converts the interfering PO^+ into free P atoms.

The sulphate interference can be overcome by the addition of $\text{Ba}(\text{NO}_3)_2$. Matrix modification is always required in order to prevent losses due to volatilisation.

1.7.7 Atomic emission spectrometry (AES) methods

A detection limit of 30 ng mL^{-1} has been reported for ICP-AES at the most sensitive 196.026 nm line. Iron can interfere with the determination and must be separated. A 50 – 100 fold increase in sensitivity and elimination of spectral interference are achieved by HG ICP AES. Systems of various degree of automation have been developed (14). Other emission techniques include hollow-cathode discharge AES and HG-DCP-AES.

1.7.8 Atomic fluorescence spectrometry (AFS) methods

Non-dispersive AFS with atomisation of H_2Se using a combined electrothermally heated quartz tube-flame atomiser offers a detection limit of 0.03 ng. Electrothermal atomic fluorescence spectrometry offers a detection limit of 15 – 80 fg and with $\text{Pd}(\text{NO}_3)_2$ as matrix modifier can be applied to various samples (14).

1.7.9 Neutron activation methods

There are only three radionuclides of selenium produced by thermal neutron irradiation, ^{75}Se , ^{77}Se , ^{81}Se , with half lives of 120 days, 15.55 and 18.6 min, respectively.

In the application of neutron activation, the unknown sample and a known weight of pure Se are exposed simultaneously to the same neutron flux. After irradiation, the sample and standard are allowed to cool for 2 weeks in order to reduce interference from short lived isotopes. For the radiochemical determination of Se, both sample and standard are digested with acid and the element is separated from the solution and precipitated by hydrazine or hydroxylamide from 8 mol L^{-1} HCl or by sulphur dioxide from 6 mol L^{-1} HCl in boiling solution. The precipitated Se is dried and weighed, and its activity is measured under the same conditions. The detection limit on ^{75}Se ranges from 0.0005 to 0.10 μg according to the sample size, neutron, flux, irradiation period and the type of radiochemical separation used (2).

1.7.10 The choice of techniques for Se determination

Haygarth et al (16) have reviewed the broad range of contemporary instrumental techniques commonly reported for the determination of total selenium. The techniques include fluorimetry, radiochemical neutron activation analysis (RNAA), hydride generation atomic spectrometry (HGAAS), both with a commercially available HG system and flow injection (FI) HG systems, HG inductively coupled plasma atomic emission spectrometry (HG-ICP-AES), and HG-ICP mass spectrometry (HG- ICP- MS). The authors conclude that fluorimetry, HGAAS and HG-ICP-AES will continue to be usefully employed for routine laboratory determination of Se, although the fluorimetric methods are becoming dated.

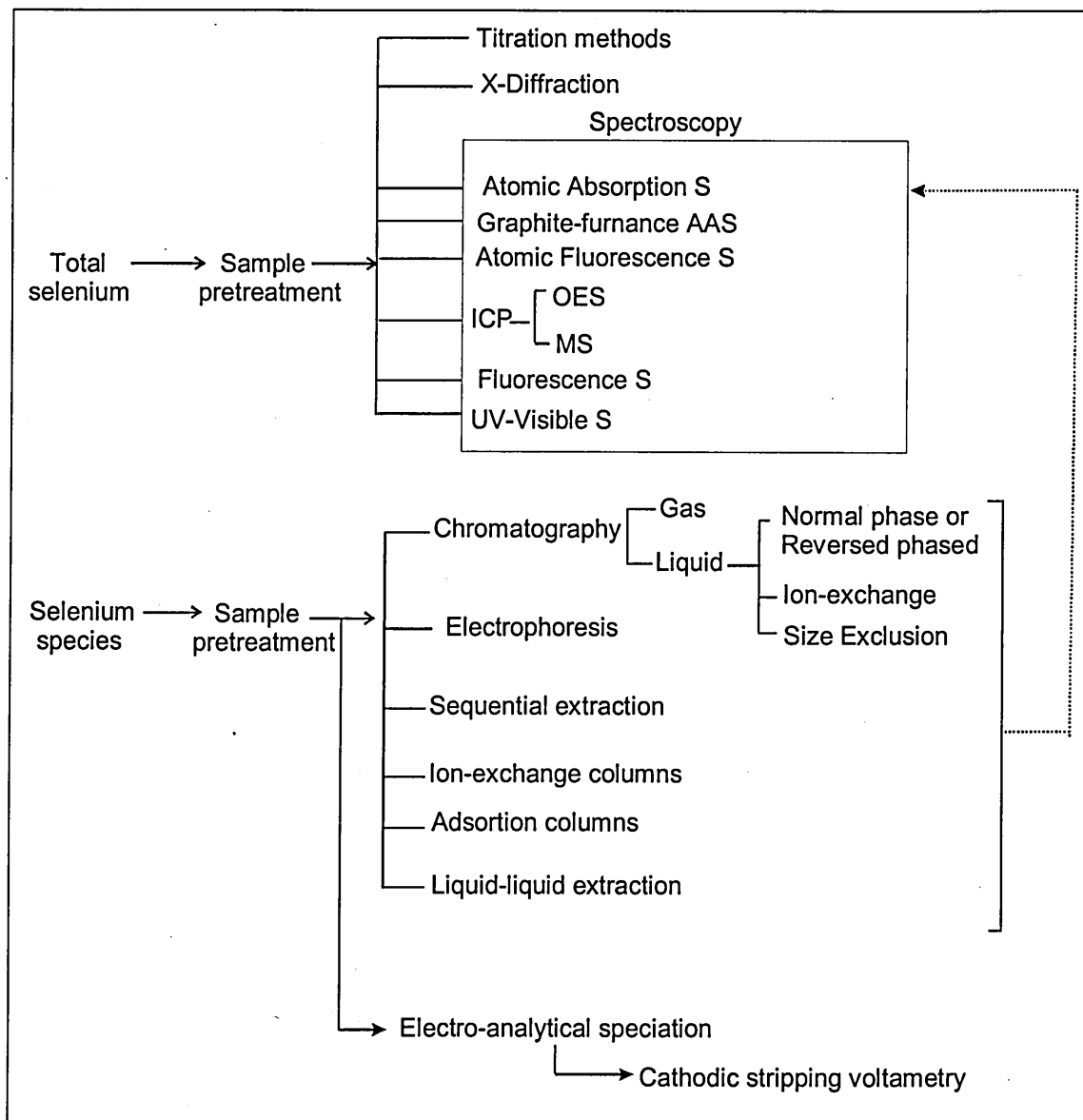


Figure 1. 3 Schematic description of the analytical methods used for selenium speciation and determination.

1.7.11 Speciation techniques

More recently, there has been a growing interest in the determination of the different selenium forms present in a sample. Speciation involves separation and determination techniques. The term speciation has often been used to indicate the analytical activity of identifying chemical species and measuring their distribution. Sometimes, it is used to indicate that a method gives more information on the form in which the element is present than other more commonly applied techniques (e.g. measuring distinct organomercury compounds as opposed to a total mercury determination). Speciation analysis is used when referring to the analytical activity of identifying and measuring species. Speciation is also used to indicate the

distribution of species in a particular sample or matrix. Chemical element speciation is related to the specific form of an element defined as isotopic composition, electronic or oxidation state, and/or complex or molecular structure. Some of the separation techniques used for chemical speciation are reviewed below.

1.7.11.1 Electrophoresis

Electrophoresis procedures separate species on the basis of their overall charge, and to a lesser extent, size. In the horizontal bed instrument, differential migration is promoted by applying an electrical potential along the length of a sheet of porous, conducting polymer, which is supported on an inert base. The ends of the bed dip into reservoirs of the buffer solution, which controls the pH and conductance of the system. When an aliquot of sample is placed on the centreline of the conducting strip, cations and anions move in opposite directions, while neutral molecules remain near the application point. With freshwater samples, for example, it was found, that simple hydrated cations such as of the alkali and alkaline earth elements moved strongly towards the cathode. High-performance capillary electrophoresis (HPCE or CE) uses a length of fused silica capillary tubing, with 25-200 μm internal diameter, in place of the porous polymer bed. The tube is fitted with an optical viewing window, and an inlet for introduction of a small plug of sample ($< 1 \mu\text{L}$). The capillary tube and end reservoirs contain an appropriate electrolyte. On application of a high potential between the ends, sample component molecules migrate at different rates along the capillary (17).

Detection of the separated species can be based on ultraviolet (UV) absorbance, which offer detection limits around $1 \times 10^{-6} \text{ mol dm}^{-3}$, or fluorescence detection, which is 100-1000 times more sensitive. Greater sensitivity and structural information can be achieved by using a mass spectrometer as the detection unit. The inherently high surface area-to-volume ratio in the capillary results in highly efficient separations of both small species as ions and organic acids and large molecules such as proteins, DNA and other natural products. Problems arising from the heat generated in the process have been minimised by circulating inert fluorocarbon coolant around the capillary (18).

1.7.12.2 Ion-exchange columns

Both anion- and cation-exchange resins can be used to separate the differently charged metal species. An example of this application is a study on natural waters, in which a cation exchange resin was used to retain selenite and selenate present in the sample, while the selenocystine was analysed (17).

1.7.11.3 Chelating resins

The introduction of chelating functional groups, as aminodiacetate, into polymer beads yields a product with a high affinity for metal ions. Compared with other techniques, such as dialysis, ultra-filtration and solvent extraction, chelating column separations can be claimed to be faster and simpler, with the equipment being relatively less expensive.

1.7.11.4 Solid-phase extraction

Columns packed with charged adsorbents, such as hydrophobic copolymers, have been used for specific separations such as isolation of trace levels of organics present in waters. Isolation and preconcentration of organic complexes of metals has been achieved through sorption on a non-polar substrate such as sep-Pak cartridges, which contain silica base material coated with a C₁₈ hydrocarbon. Uncomplexed metal ions can be partially sorbed, and retained species are retrieved through elution with organic solvents, although it has been noted that in some systems such as seawater, the recovery of metal organics is not always quantitative.

1.7.11.5 Liquid-liquid extraction

When an aqueous sample is shaken with a volume of immiscible organic solvent, non polar species tend to be extracted into the organic layer. This liquid-liquid extraction procedure has some applications in speciation studies. The liquid-soluble fraction of the metal has been extracted from natural waters using solvent mixtures such as n-hexane, 10% butanol and n-octanol and 20% butanol in hexane. The liquid-liquid extraction efficiencies are low, because of poor transfer of the charged complexes between phases. In addition, species adsorbed on organic colloids are partially extracted.

1.7.11.6 Chromatographic methods

Open column liquid chromatography

In open column liquid chromatography, the sample is added to the top of a column packed with adsorbent material such as alumina, silica gel, polymer gel or a fine particle substrate coated with an organic compound. Differential movement of the sample components is then promoted by gravity feeding of the eluent, which could be aqueous or organic, through the column. The separated components are identified by analysis of eluted fractions. This technique has been used to separate both volatile and non-volatile inorganic species but the procedure is time consuming (19).

High-performance liquid chromatography

In this technique, small samples, 50 μL or less, are introduced into an injection valve and passed through a column packed with fine adsorbent. The liquid eluent is pumped through the column at a rate of mL min^{-1} . With suitable packings the components of the sample can be separated in a few minutes. To achieve separations using different types of samples, the nature of both the stationary and mobile phases can be varied. In normal phase chromatography, the stationary phase is usually silica or alumina, which have high polarity, and the eluent is a volatile non-polar solvent. Speciation applications are mainly limited to the separation of stable neutral chelates such as metal complexes containing dithiocarbamate, dithizonate, thio-oxinate, thionate or β -diketonates. In reversed-phase HPLC, the stationary phase is composed of a non-polar material such as octadecylsilane, which is a C_{18} substituted bonded silica, and the eluent is a polar solvent. In addition to separation of metal chelates and organo-metallic species, reversed-phase operation can be used to separate inorganic cations and anions, but in this application the mobile phase contains a charge-neutralising species such as n-pentane sulphonate or tert-butylammonium phosphate (19).

HPLC units have been interfaced with a wide range of detection techniques: spectrophotometry, fluorimetry, refractive index measurement, voltammetry and conductance, but most of these only provide partial information about the nature of the chemical species. As with other forms of chromatography, for component identification, the retention parameters have to be compared with the behaviour of known chemical species. For organometallic species element-specific detectors such as spectrometers, which measure atomic absorption, atomic emission and

atomic fluorescence, have proved quite useful. The ideal HPLC detection system for cations is an ICP-MS unit (18).

For anions, the separation column is packed with pellicular strong base anion-exchange resin. Ion chromatography is used mainly for inorganic ions such as selenite and selenate, but separations of other anions and selenium organics such as selenomethionine, and other seleno forms have also been achieved (18).

Gas chromatography

GC has been used extensively for the separation and determination of volatile organic molecules in the inorganic trace analysis area. However, few species possess the required volatility, and applications tend to be limited to the separation of volatile species of low molecular weight such as methyl derivatives of As, Se, Sn, Hg and the separation of semi-volatile organometals. Hydride generation (HG) has been reported for the generation of volatile forms of selenium. For organo-metal species, the type of detection system required varies with the nature of the analyte, and the options include electron capture detection, flame photometric detection, ICP, AAS and MS (18, 20).

1.7.11.7 Electro-analytical speciation techniques

Electro-analytical techniques have been used extensively for natural waters. For example, ion-selective electrodes allow measurement of the activity of free hydrated ions in solution, which are species highly relevant to toxicity studies, and voltammetric methods, such as polarography and ASV, exhibit a high degree of selectivity for highly labile species. Electro-chemical techniques also facilitate identification of the valency state of elements such as Fe, Cr, Tl, Sn, Mn, Sb, As, Se, V, U and I. For several other elements only one state is electro-chemically active, and redox state speciation becomes a special case of labile/inert species discrimination.

Cathodic stripping voltammetry

In cathodic stripping voltammetry an insoluble film, which is usually the mercury salt of the analyte anion, is deposited on the working electrode by application of a positive potential. The salt is then displaced by a cathodic stripping cycle, by applying a reagent electrode potential. Applications include determination of sulphide present in a large excess of other sulphur compounds, and determination of As (III) or Se (IV) in the presence of their higher valency states. In another version

of the technique, a thin film of organic ligand is collected on the working electrode, prior to sample introduction. Trace elements present in the sample interact with the adsorbed ligand to form metal complexes. The electrode is then subjected to a cathodic sweep operation and reduction of the surface-active metal species, to form a metal amalgam, yielding a current flow, which is a sensitive measure of the initial trace element content (18).

1.8 TECHNIQUES USED THROUGHOUT THIS RESEARCH

1.8.1 Purification

After a product has been isolated from a reaction the next step is its purification. The degree of purity required depends on the use for which the sample is intended: a synthetic intermediate might only require rough purification, whereas a product for elemental analysis would require rigorous purification. The most important purification techniques are crystallisation, distillation, sublimation and chromatography.

1.8.1.1 Crystallisation

Simple recrystallisation of an impure solid is a routine operation, which nevertheless requires care and good judgement if good results are to be obtained. The basic procedure involves six steps, which are: selection of a suitable solvent, dissolution of the compound in the minimum volume of hot solvent, filtration of the hot solution, crystallisation, and filtration to remove the crystals.

The most commonly used solvents in order of increasing polarity are petroleum ether, toluene, chloroform, acetone, ethyl acetate, ethanol, and water. Chloroform and dichloromethane are rarely useful on their own because they are good solvents for the vast majority of organic compounds. It is preferable to use a solvent with a boiling point in excess of 60 °C, but at least 10 °C lower than the melting point of the compound to be crystallised, in order to prevent the solute from 'oiling out' of solution. In many cases, a mixed solvent can be used, and combinations of toluene, chloroform, or ethyl acetate, with the petroleum ether fraction of similar boiling point are particularly useful.

Once the solvent has been chosen the crude sample is dissolved in a minimum volume of hot solvent in a conical flask fitted with a reflux condenser. If a mixed

solvent is used, dissolve the crude in a small volume of the good solvent, heat to reflux, add the poor solvent in portions until the compound just begins to precipitate (cloudiness), add a few drops of the good solvent to redissolve the compound, and allow to cool.

Filtration of the hot solution to remove insoluble impurities is often problematic, and should not be carried out unless an unacceptable amount of insoluble material is suspended in the solution. The difficulty here is that the compound tends to crystallise during filtration so an excess of solvent should be added, and the apparatus used for the filtration should be preheated to about the boiling point of the solvent. If the solution is very dark and/or contain small amounts of impurities, it should be allowed to cool for a few minutes before adding 2% by weight of decolourising charcoal, heating to reflux for a few minutes, and filtering off the charcoal. Very dark or tarry products are chromatographed through a short (2-3cm) plug of silica before recrystallisation is attempted.

Crystallisation is usually straightforward except when the material is very impure or has a low melting point ($< 40\text{ }^{\circ}\text{C}$) in which case it sometimes precipitates as an oil. If an oil forms it is best to reheat the solution and then to allow it to cool slowly. Scratching the flask with a glass rod or adding a few 'seed' crystals to induce crystallisation might help to start the process, and if this fails the addition of more solvent so that precipitation can occur at a lower temperature. If both fail, then scratching with a glass rod, seeding, or cooling the solution in ice-water can be tried. If all the above fail, the setting aside the flask for a few days usually works.

When crystallisation is complete the crystals are filtered using an appropriately sized sintered glass funnel. It is very important to wash the crystals carefully. As soon as all of the mother liquor has drained through the funnel, suction is stopped and cold solvent is poured over the crystals, stirring them if necessary, in order to ensure that they are thoroughly washed. The washings are drained off under suction and the process is repeated once or twice more. After careful washing, the crystals are allowed to dry briefly in air. The last traces of solvent are removed under vacuum. The crystals should be protected against accidental spillage or contamination (21).

1.8.1.2 Open Column

Chromatography techniques can be used to purify small quantities of material. Traditional open column or flash column chromatography can be used for the purification. In this type of purification the glass column is filled with the required adsorbent, which is typically silica gel. The amount of adsorbent used depends upon the quantity of crude sample to be purified. However, it is inadvisable to fill the column to more than three-quarters full, otherwise there is insufficient room for the solvent. Next, the eluting solvent is added to the top of the column and allowed to run through the column under gravity. More solvent is added to the top of the column as required. Once the solvent starts to appear at the bottom of the column, the fractions can be collected (17, 21)

1.8.2 Microwave digestion

Microwave heating involves direct absorption of energy by the sample material being digested. Microwaves are electromagnetic energy, which is a non-ionising radiation that causes molecular motion by the migration of ions and rotation of dipoles, but does not cause changes in molecular structure. Microwave energy has a frequency range between 300 to 300 000 MHz. The laboratory microwave cavity system is based on commercial microwave equipment. However, the resonant cavity and other components of these devices are similar to those of a domestic microwave oven but the laboratory unit is equipped with numerous safety features. The microwave system must be capable of venting the fumes without contacting or damaging the control electronics. To prevent acid corrosion, the inner walls of the microwave cavity are made from resistant materials such as stainless steel covered with Teflon. Laboratory microwave units are also equipped with inter-locking door mechanisms, explosion-resistant doors with resealing hinge systems and door shields. These design considerations differ significantly from those used in domestic microwave ovens.

Microwave digestion systems also offer additional advantages over pressurised bombs with faster digestion times and more controlled analysis with high reproducibility. A digestion bomb is a moderate or high pressure microwavable vessel without temperature or pressure measurement connection capabilities. A digestion vessel however, has the capability to connect to at least one reaction condition monitoring system and usually has an excess pressure relief system (22).

1.8.3 Inductively coupled plasma

Inductively Coupled Plasma (ICP) is one of the most widely used excitation sources used for atomic emission spectrometry. An ICP allows rapid multi-element determinations for different elements in a wide range of concentrations. When atoms or ions are excited, they emit discrete radiation which is characteristic of the type of atom examined. Emission signals from ions or atoms generated in plasma are measured (23).

A typical ICP source, known as a torch, consists of concentric quartz tubes through which argon flows (see Figure 1. 4). Surrounding the top of the outer tube is an induction coil that is energised by the radio frequency generator creating a changing magnetic field. To initiate the ionisation of the flowing gas, a discharge from a Tesla coil or a pilot spark is applied. Ions and their associated electrons interact with the fluctuating magnetic field generated by the induction coil. The inductively coupled argon plasma (ICAP or ICP) torch derives its sustaining power from the interaction of a high-frequency magnetic field and outer two quartz tubes of the torch assembly. Radio-frequency power (27 to 30 MHz) is applied through the induction coil. This sets up an oscillating magnetic field. Power transfer between the induction coil (two-turn primary winding) and the plasma (one-turn secondary winding) is similar to a power transfer in a transformer (23, 24). The resistance to the movement of the ions and electrons produces heat. Temperature of up to 10 000 K can be achieved in the plasma. Thermal isolation is achieved by flowing argon tangentially around torch walls.

The light emitted is dispersed by a grating or prism monochromator. The spectral lines produced are recorded either on a photographic plate, or, in modern systems, by diode arrays or photomultiplier tubes linked directly to computer-controlled data-processing systems.

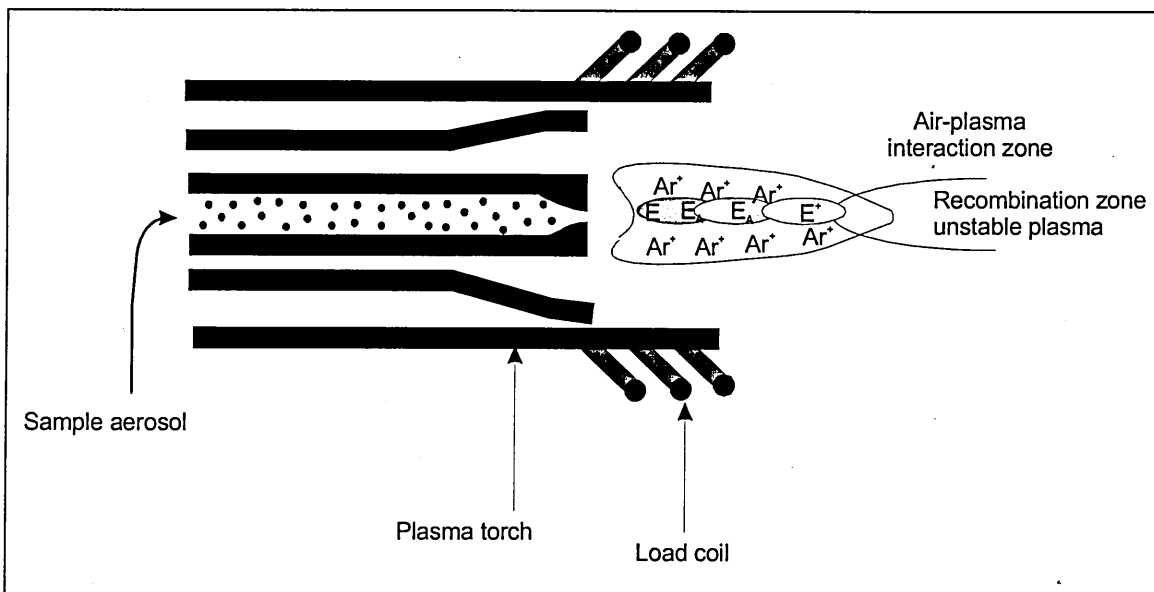


Figure 1. 4 Schematic view of a horizontal Ar - ICP torch for ICP-AES and ICP-MS.

1.8.3.1 ICP-MS

Inductively coupled plasma mass spectrometry (ICP-MS) combines an ICP, as a high temperature ion source with a quadrupole mass spectrometer (MS), as a highly sensitive detector. Mass spectrometric analysis involves the separation of the ions on the basis of their atomic mass-to-charge ratio (m/z). Over the past decade there has been a significant increase in the use of ICP-MS for multi-element analysis because of its low detection limits for most all elements and isotopic analysis capacity (24, 25). The disadvantages of ICP-MS compared with ICP-AES includes; higher instrument costs, instrument drift that can be range between 5 - 10% per hour, and certain interferences.

Once ions have been formed in the plasma, they are transmitted through the sampling cone, which is a water-cooled nickel cone with a central 1mm aperture. A fraction of the gas in this region then passes through a small hole in a second cone called a skimmer. The pressure between the cones is maintained at 100Pa by a rotary vacuum pump and is reduced continuously from behind the skimmer cones to the analyser chamber (0.1Pa). This allows continuous sampling of the bulk plasma. The gas coming into the first low-pressure stage expands depending upon the aperture size and sampling-skimmer cones distance. The bulk of the gas is pumped away by the rotary vacuum pump and a small fraction passes through the final aperture into the quadrupole mass analyser. The omega lens system, which is located before mass spectrometer, serves to focus ions coming from the skimmer into the mass filter rejecting neutral atoms and minimising photons from plasma. The

quadrupole consists of four parallel cylindrical rods that serve as electrodes. In the quadrupole, all the ions are converted to neutral molecules except those having a certain mass-to-charge ratio (m/z) that are allowed to pass through and reach the transducer or detector at any point in time. The detector measures the ion signal at each mass and stores it. Data is expressed as counts per second, which are directly proportional to the concentration of the element at that mass.

The occurrence of interferences limits the application of ICP-MS. Mass spectroscopic and non spectroscopic interferences can occur. The former includes inter-element (e.g. ^{65}Zn and ^{65}Cu), poly-atomic (e.g. $^{80}\text{Ar}_2^+$ and ^{80}Se), and doubly-charged ions interferences (e.g. $^{88}\text{Sr}^{+2}$ and ^{44}Ca). The non-spectroscopic interferences are caused by high levels of dissolved solid, high mass elements, and easily ionised elements in the sample matrix. Sample dilution, instrument optimisation, internal standardisation, standard additions and matrix elimination are common procedures used to limit or eliminate both spectroscopic and non-spectroscopic interferences (26).

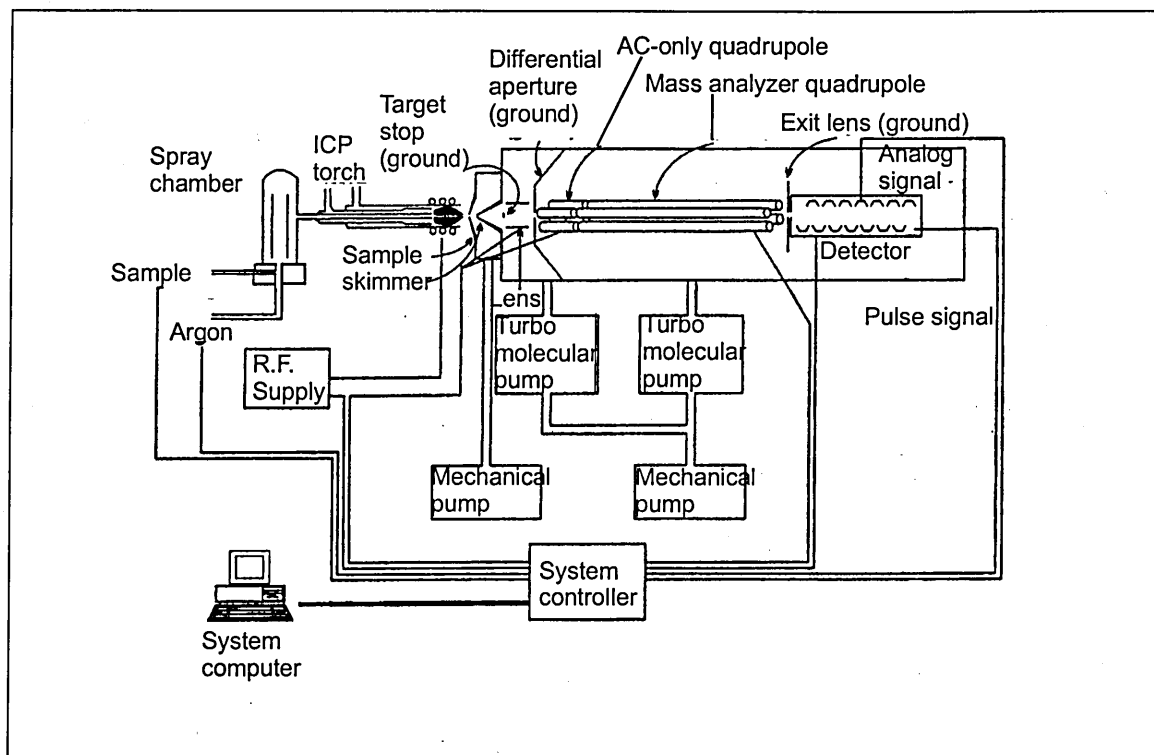


Figure 1. 5 Schematic diagram of a quadrupole ICP-MS (24).

1.8.3.2 Liquid chromatography coupled to inductively coupled plasma-mass spectrometry

Improvements in instrumental performance and design, together with the development of new methods, have resulted in an increased demand for more

sensitive and reliable techniques in trace element speciation. The coupling of an LC to ICP-MS is relatively simple. The compatibility of flow rates enable the LC column to be directly connected to the ICP nebuliser using a length of tubing, which must be kept as short and narrow as possible to minimise dead volume and band broadening. The spray chamber is used to sort the aerosol droplets according to size and generally, only about 2% of the sample solution, passing through the concentric nebuliser actually reaches the plasma. A typical LC-ICP-MS set-up is shown in Figure 1. 6.

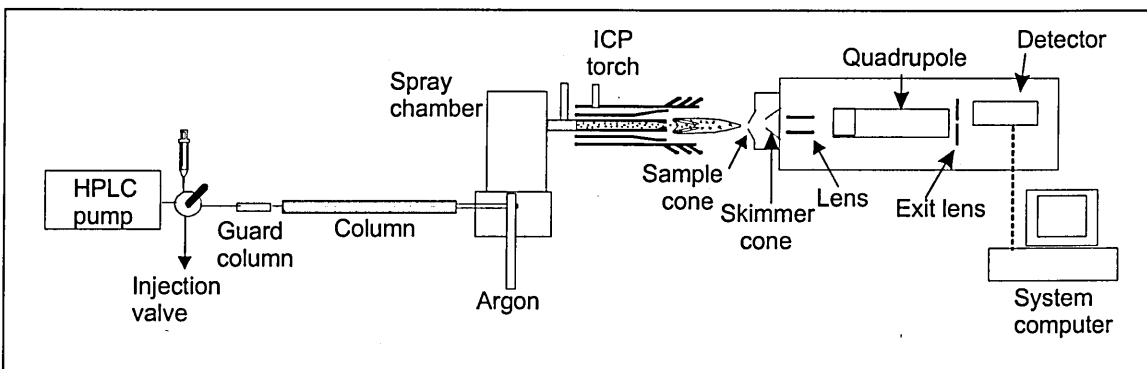


Figure 1. 6 Schematic diagram of HPLC-ICP-MS system

Other nebulisers, such as the glass frit, the ultrasonic and the direct injection type, increase efficiency but require lower flow rate.

When coupling LC to ICP-MS several factors need to be taken into account. The salt content of the mobile phase must be kept to a minimum (<2%), to prevent clogging of the nebuliser and erosion of the sampler and skimmer cones, which often cause a change to the width of ion input aperture. Mobile phases which contain a large amount of organic solvent may cause plasma instability and may ultimately quench it. Another effect of using high organic content mobile phases is the build up of carbon as soot on the sampler cone, which will cause a decrease in the analyte signal. A possible solution is to cool the spray chamber in order to prevent large amounts of organic solvent vapour from entering the plasma. Desolvation and the addition of oxygen to the nebuliser provide better solutions. The forward power of the plasma can be increased to aid the decomposition of the organic components; however, this may be accompanied by an increase in reflected power, which could harm the generator (27). The use of hydride generation coupled to a HPLC and to ICP-MS help eliminate matrix interferences and leads to improvements in detection limits.

1.8.3.3 Hydride generation

Hydride generation has been coupled to AAS, ICP-AES and ICP-MS for the determination of volatile hydrides such as those of arsenic, bismuth, germanium, lead, antimony, selenium, tin and tellurium. In contrast to solution nebulisation, the Se volatile hydrides are more efficiently transported to the plasma, where ions are produced for mass spectrometry. In addition, the resulting separation of analytes from the sample matrix and the solvent reduces potential spectral interferences and enables preconcentration of the analytes. One problem inherent to hydride generation is that the reaction by-products such as H_2 , CO_2 , and H_2O , can cause instability or even extinguish the plasma. The hydride generator, in general, includes a peristaltic pump connected through a four-way valve to the gas-liquid separator. This design enables the sample solution to be rapidly switched with the blank, in effect providing a full analysis, while completely excluding the injection of an air plug into the plasma. With this modification, the system stability has been markedly improved, and the ICP can be operated at a much lower power (1.25 kW).

Design refinements to the gas-liquid separator reduce the power needed to maintain a stable plasma and the quantities of reagents required. The addition of NaOH solution to the sample waste neutralises liquid by-products and reduces the amount of H_2 evolved. Excess H_2 reaching the plasma can be reduced by continuously pumping the reaction by-products from a combination of reaction-vessel gas-liquid separator. Microporous polytetrafluoroethylene (PTFE) membranes and tubing have been investigated as phase separators. Membrane separators rapidly separate hydrides from the reaction solution, thus reducing the interferences from argon polyatomic species on As and Se. Commercial hydride generators with a membrane gas-liquid separator have been developed, leading to improvements of detection limits of 50- to 100-fold compared with solution nebulisation for As, Se, Sn, Sb, Ge, and Hg. Hydride generation in a batch system as well as on-line has also been used. The batch generation of hydrides usually involves rapid mixing of the acid and the borohydride solutions in a reaction vessel followed by condensation of the volatile hydrides in a liquid argon trap, the hydrogen created in the process is removed and the hydrides are then revolatilised by placing the condensation trap in a hot water bath. Ar gas sweeps the gaseous hydrides into the plasma. However, hydride generation by flow injection offers rapid sample throughput, ease of automation, and the feasibility of using small sample volumes between 20 to 200 μL . A general flow injection system for HG is shown in Figure 1. 7.

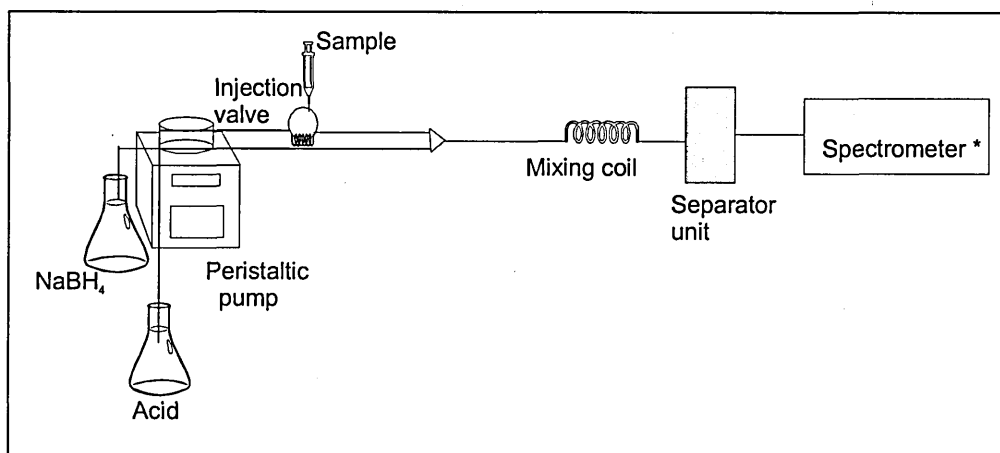


Figure 1. 7 A schematic diagram of hydride generation system using flow injection, * with either AAS, AFS, ICP-EOS, or ICP-MS detection.

Hydride generation post column derivatisation can be used for HPLC-ICP-MS after which the hydride is transported to the plasma. A glass gas-liquid separator unit is used in place of the conventional nebuliser-spray chamber because of a significant memory effect with the latter. Detection limits are sufficiently improved to permit its application to many complex samples.

The major disadvantage of most hydride generation techniques, including the couple used with the ICP-MS system is the inability to simultaneously determine other volatile-hydride-forming elements. Other problems commonly associated with the coupling between HG and ICP-MS are high blanks, memory effects and plasma instability. The design of the gas-liquid separator is a key factor affecting all three parameters (27).

1.8.4 Chromatography

To achieve chromatographic separation, the sample mixture is distributed between the mobile and stationary phase. The stationary phase is either a solid or a thin film of liquid coated on a solid support or column wall. The mobile phase is usually a gas or liquid. If a gas is used as a mobile phase, the process is known as gas chromatography (GC); when the mobile phase is liquid the separation is known as liquid chromatography (LC). High performance liquid chromatography (HPLC) was derived from the classical column chromatography and, besides gas chromatography, is one of the most important tools of modern analytical techniques (28). Liquid chromatographic methods are commonly categorised into different types based upon the nature of the stationary phases and the kinds of equilibria involved in the transfer of solutes between the mobile and stationary phases (19).

Figure 1. 8 shows the different types of LC that have been developed.

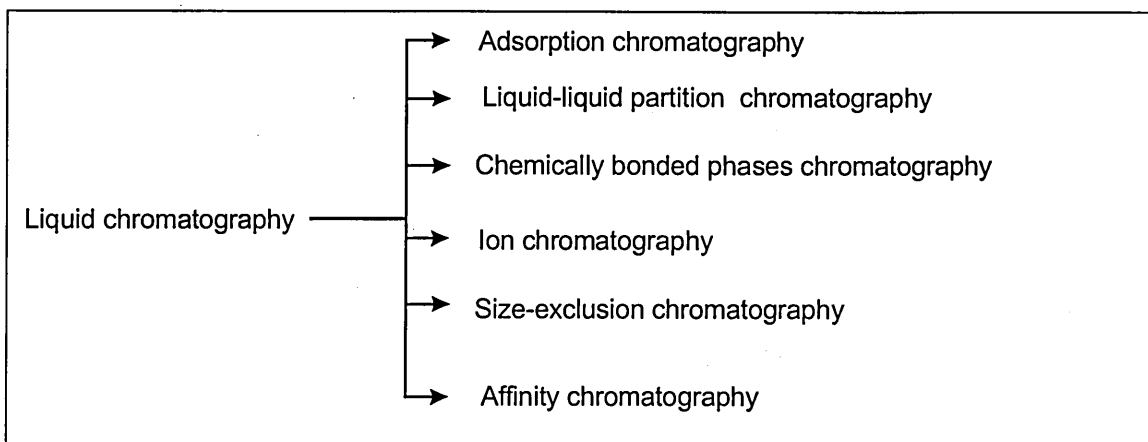


Figure 1. 8 The different modes of liquid chromatography.

1.8.4.1 Ion Chromatography

Types of Ion Chromatography

Ion-Exchange Chromatography (High Performance Ion Chromatography, RPIC)

This separation method is based on ion-exchange processes occurring between the mobile phase and ion-exchange groups bonded to the support material. Additional non-ionic adsorption processes contribute to the mechanisms involved in the separation of highly polarisable ions. The stationary phase consists of a polystyrene resin copolymerised with divinylbenzene and modified with ion-exchange groups. Ion-exchange chromatography is used for the separation of both organic and inorganic anions and cations, respectively. Separation of anions is accomplished with quaternary ammonium groups attached to the polymer, whereas sulfonate groups are used as ion-exchange sites for the separation of cations.

Ion-Exclusion Chromatography (High Performance Ion Chromatography Exclusion, UPICE)

The separation mechanism in ion-exclusion chromatography is governed by Donnan steric exclusion, and sorption processes. A totally sulphonated polystyrenedivinylbenzene-based cation exchange material with high capacity is employed as the stationary phase. Ion-exclusion chromatography is particularly useful for the separation of weak inorganic and organic acids. In combination with suitable detection systems, this separation method is also useful for the determination of amino acids, aldehydes, and alcohols.

The dominating separation mechanism in ion-pair chromatography is adsorption. The stationary phase consists of a neutral porous divinylbenzene resin of low polarity and high specific surface area. Alternatively, octadecylsilane, (C_{18}), can be used. The selectivity of the separation column is determined solely by the mobile phase. Besides an organic modifier, an ion- pair reagent is added to the eluent (water, aqueous buffer solution, etc.) depending on the chemical nature of the analytes.

In addition to the three classical separation methods mentioned above, reversed-phase liquid chromatography (RPLC) is becoming increasingly popular for the separation of highly polar and ionic species, respectively. This separation mode is known as ion suppression. Chemically bonded aminopropyl phases have also been successfully employed for the separation of inorganic ions (18)

1.8.5 Flow injection analysis

Flow injection analysis (FIA) is based on the injection of a liquid sample into a moving, non-segmented continuous carrier stream of a suitable liquid. The injected sample forms a zone, which is then transported toward a detector that continuously records the signal (29). FIA was developed by Ruzicka and Hansen in the mid 1970s. This technique allows easy sample and reagent mixing offering high analysis rates, in the range of 100 to 300 samples hour⁻¹. The advantages of FIA are the increase of response times together with much more rapid start-up and shut-down times, (less than 5 min). These characteristics make it easy to use and economical for automated measurements. A simple type of FIA system is composed of a peristaltic pump, an injection valve, reactor coil and a detector as shown in Figure 1. 9.

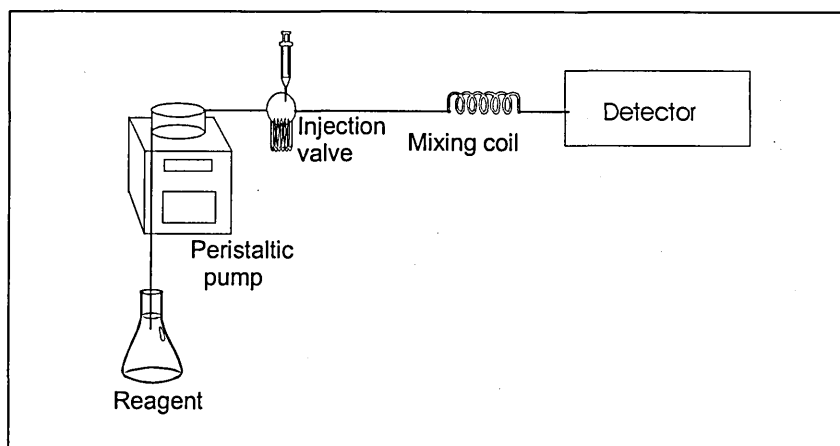


Figure 1. 9 A schematic diagram of a simple flow injection system.

For a successful analysis, it is vital that the sample solution is injected rapidly as a pulse or plug of liquid, and, in addition the injection must not disturb the flow of the carrier stream.

When a sample is injected into the system it moves through the tubing and as a consequence, band broadening or dispersion takes place. The shape of the resulting peak is determined by convection arising from laminar flow in which the centre of the fluid moves more rapidly than the liquid adjacent to the walls. As a result, a parabolic shape or a skewed zone profile shown Figure 1. 10 is observed.

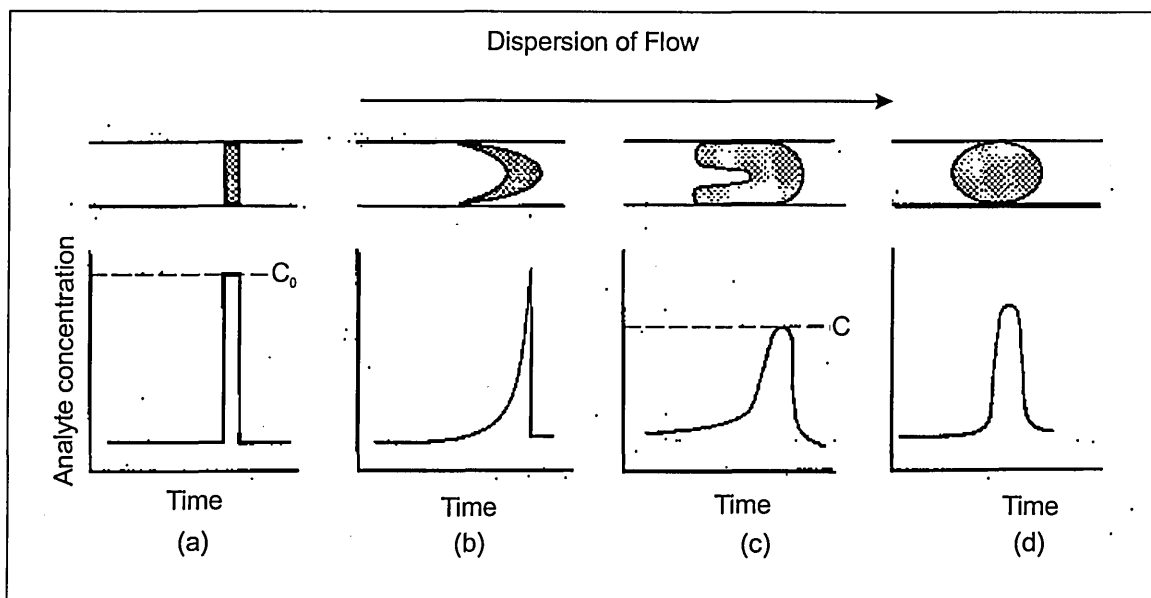


Figure 1. 10 Effect of convection and diffusion on concentration profiles of analytes at the detector: (a) no dispersion, (b) dispersion by convection, (c) dispersion by convection and radial diffusion, and (d) dispersion by diffusion (30)

The other factor that affects peak shape is diffusion, which could be radial, perpendicular or parallel to the direction of flow. It has been shown that radial diffusion is always important, while the longitudinal diffusion is not significant in

narrow tubing. Dispersion increases at low flow rates and broad peaks are obtained as shown in Figure 1. 10.d. Dispersion is defined by the equation, $D = C_0/C$ where C_0 is the analyte concentration of the injected sample and C is the peak concentration at the detector. Dispersion is influenced by three variables: sample volume, tube length, and flow rate. The effect of sample volume is shown Figure 1. 11.a. As shown in Figure 1. 11.b, dispersion is proportional to the tube length.

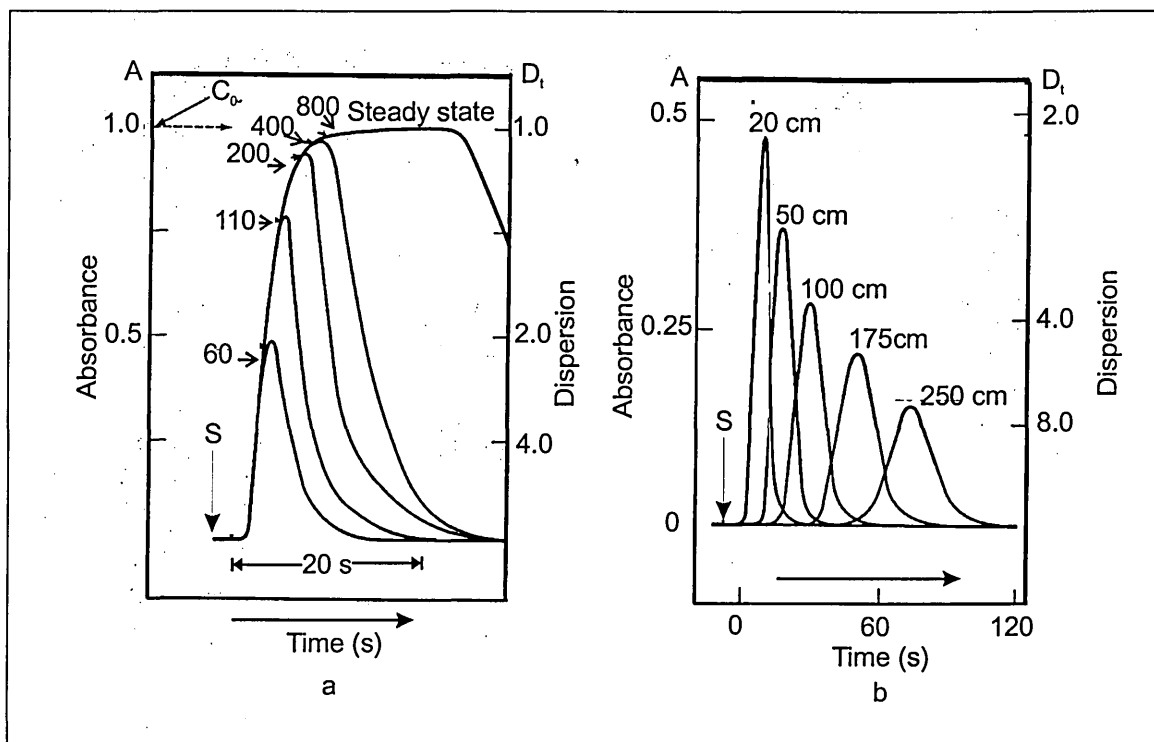


Figure 1. 11 The effect of sample volume and length of tubing on dispersion. (a) shows the signal obtained when the tube length was kept at 20 cm; flow rate: 1.5 mL min⁻¹; and the injected volume varied between 60 to 800 µL. (b) shows the signal obtained when the sample volume was kept at 60 µL; flow rate: 1.5 mL min⁻¹ and the tube length varied between 20 to 250 cm(30).

Dispersion is inversely proportional to flow rate (30). Methods based on limited, medium, and large dispersion have been developed. Limited dispersion flow injection techniques have been applied to detectors such as flame atomic absorption and emission, as well as inductively coupled plasma atomic emission spectrometry. With those instruments sample aliquots are aspirated directly into the flame or plasma, and a steady-state signal is measured. Under flow injection conditions, in contrast, a blank reagent is pumped through the system to the detector continuously to give a baseline output; samples are injected periodically, and the resulting transient analyte signals are recorded. Sampling rates up to 300 samples per hour have been reported. Limited dispersion has been also used with specific ion and voltammetric electrodes as detectors, for which a small sample size (25 µL) and a

short measurement time, could be used. Medium dispersion systems have been applied to colorimetric determinations. Those manifolds are generally more complex. Indeed, a medium dispersion system usually includes mixing coils, separator units before the detector, all of which increase the system dispersion. In stopped flow methods, using small diameter tubings, dispersion decreases with flow rate. It has been found that dispersion decreases when flow is stopped. Stopped flow is used for two reasons: to increase the sensitivity of measurements by increasing the residence time (t) and thus the conversion of the measured species, and also to record a reaction rate which then serves as the basis for obtaining the analytical result. In this application, the flow is stopped with the reaction mixture in the flow cell where changes in the concentration of reagents or products can be followed as a function of time (29, 30).

1.8.6 Molecular fluorescence

Fluorescence and phosphorescence are alike in that excitation is brought about by absorption of photons. As a consequence, the two phenomena are often referred to by the more general term photoluminescence. Fluorescence differs from phosphorescence in that the electronic energy transitions responsible for the fluorescence do not involve a change in electron spin. As a consequence, fluorescence is short-lived, with luminescence ceasing almost immediately ($< 10^{-5}$ sec). In contrast, a change in electron spin accompanies phosphorescence emissions, which causes the radiation to endure, often for several seconds or longer after termination of irradiation. Photoluminescence emission is longer in wavelength than the radiation used for its excitation. A third type of luminescence process is chemiluminescence, which is based on the emission spectrum of an excited species that is formed in the course of a chemical reaction. In some instances, the excited species is the product of a reaction between the analyte and a suitable reagent, which is usually a strong oxidant such as ozone or hydrogen peroxide, and the result is a spectrum characteristic of the oxidation product of the analyte or the reagent rather than the analyte itself. In other instances, the analyte is not directly involved in the chemiluminescence reaction; instead, the inhibiting or the catalytic effect of the analyte on a chemiluminescence reaction is measured. Measurement of the intensity of photoluminescence or chemiluminescence permits the quantitative determination at trace levels of a variety of important inorganic and organic species. One of the most attractive features of luminescence methods is their inherent

sensitivity, with detection limits often being one to three orders of magnitude lower than those for absorption spectroscopy (31).

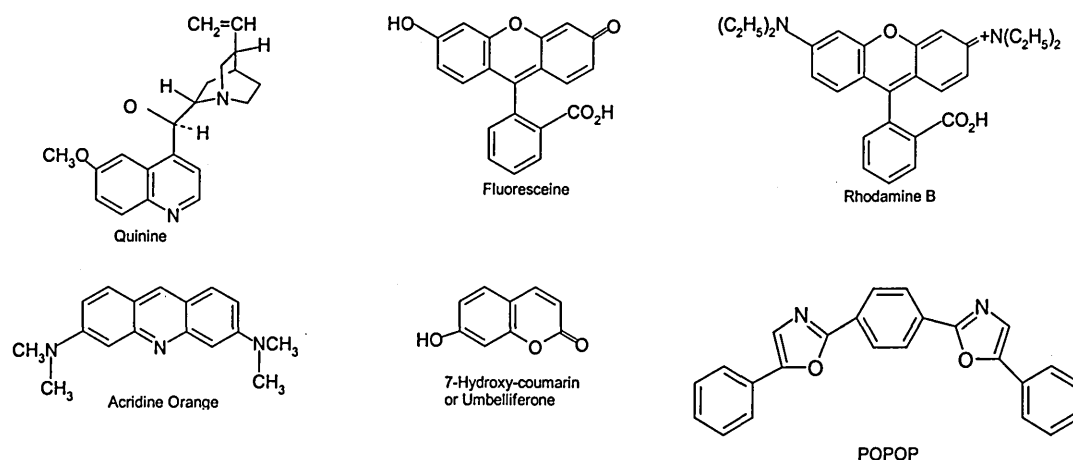


Figure 1. 12 Common fluorogenic compounds used for Fluorescence detection (32)

Fluorescence information is generally presented in the form of an emission spectrum. A fluorescence emission spectrum is a plot of the fluorescence intensity versus wavelength (nanometers) or wavenumber (cm^{-1}). Two typical fluorescence emission spectra are shown in Figure 1. 13. The spectra vary widely and are dependent upon the chemical structure of the fluorophore and the solvent in which it is dissolved. The process, which occurs, between the absorption and emission of light are usually illustrated by a Jablonski diagram. A typical Jablonski diagram is shown Figure 1. 14. S_0 , S_1 , S_2 , denote singlet ground, first and second electronic states, respectively. At each of these electronic energy levels the fluorophores can exist in a number of vibrational energy levels, denoted by 0, 1, 2 etc. The first step is the absorption of energy required for the change from S_0 to S_1 . This energy is provided by UV light. Following light absorption, several processes usually occur. A fluorophore is usually excited to higher vibrational levels, either S_1 or S_2 . With a few rare exceptions molecules in condensed phases rapidly relax to the lowest vibrational level of S_1 . This process is called internal conversion and generally occurs in 10^{-12} s or less. Since fluorescence lifetimes are typically near 10^{-8} s, internal conversion is generally complete prior to emission. Hence, fluorescence emission generally results from a thermally equilibrated excited state, that is, the lowest energy vibrational state of S_1 .

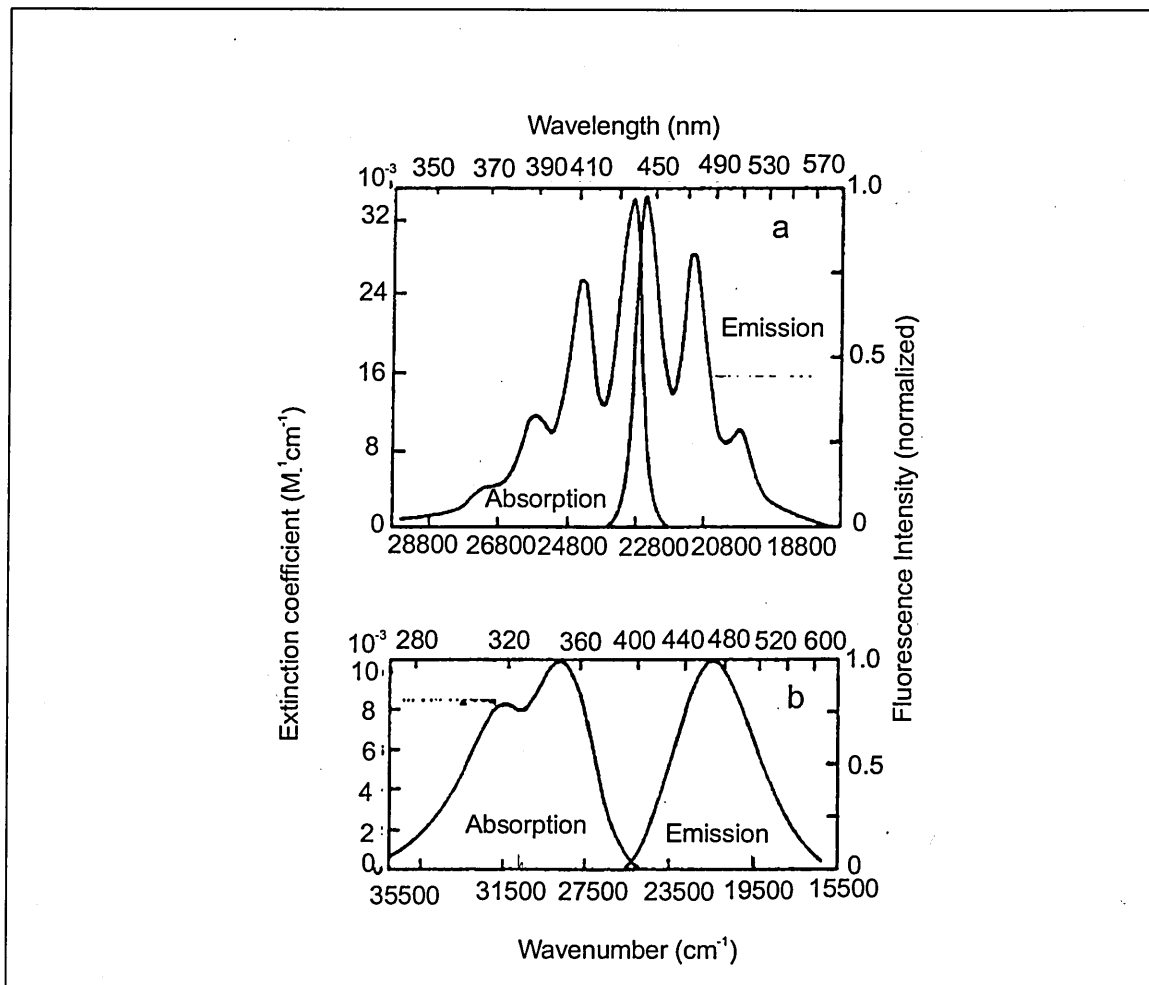


Figure 1. 13 Absorption and fluorescence emission spectra of (a) perylene and (b) quinine (32)

Return to the ground state typically occurs via a higher excited vibrational ground-state level, which then quickly (10^{-12} sec) reaches thermal equilibrium. An interesting consequence of emission to higher vibrational ground states is that the emission spectrum is typically a mirror image of the absorption spectrum of the $S_0 \rightarrow S_1$ transition. This similarity occurs because electronic excitation does not greatly alter the nuclear geometry. Hence, the spacing of the vibrational energy levels of the excited states is similar to that of the ground state. As a result, the vibrational structures seen in the absorption and the emission spectra are similar (see Figure 1. 13).

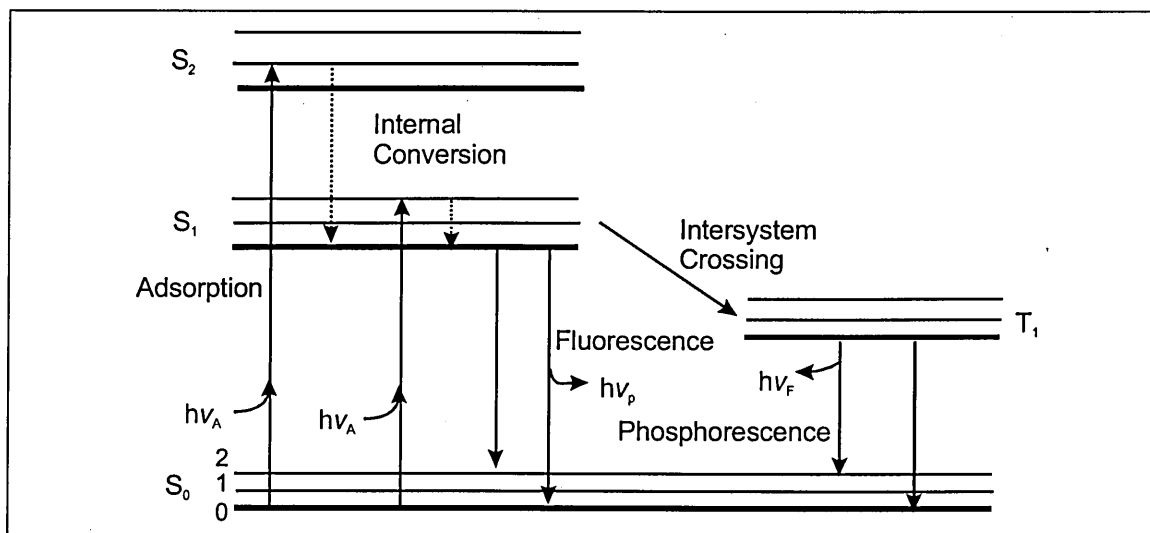


Figure 1. 14 Typical Jablonski diagram (32).

1.8.6.1 Fluorescence Quenching

The intensity of fluorescence can be decreased by a wide variety of processes. Such decreases in the intensity are called quenching. Quenching can occur by different mechanisms. Collisional quenching occurs when the excited-state fluorophore is deactivated upon contact with other molecule called the quencher (32).

1.8.6.2 Instrumentation for fluorescence spectroscopy

The components of instruments for measuring photoluminescence are similar to those found in ultraviolet/visible photometers or spectrophotometers. Nearly all fluorescence instruments employ double-beam optics in order to compensate for fluctuations in the source power. The sample beam first passes through an excitation filter or a monochromator, which transmits radiation that will excite fluorescence but excludes or limits radiation of the wavelength of the fluorescence emission. Fluorescence is propagated from the sample in all directions but is most conveniently observed at right angles to the excitation beam; at other angles, increased scattering from the solution and the cell walls may cause large errors in the measurements of intensity. The emitted radiation reaches a phototransducer after passing through a second filter or monochromator that isolates the fluorescence for the measurement. The reference beam passes through an attenuator that reduces its power to approximately that of the fluorescence radiation. The signal from the reference and sample photomultiplier tubes are then fed into a

difference amplifier whose output is displayed by a meter or recorder. A typical fluorescence instrument is shown in Figure 1. 15.

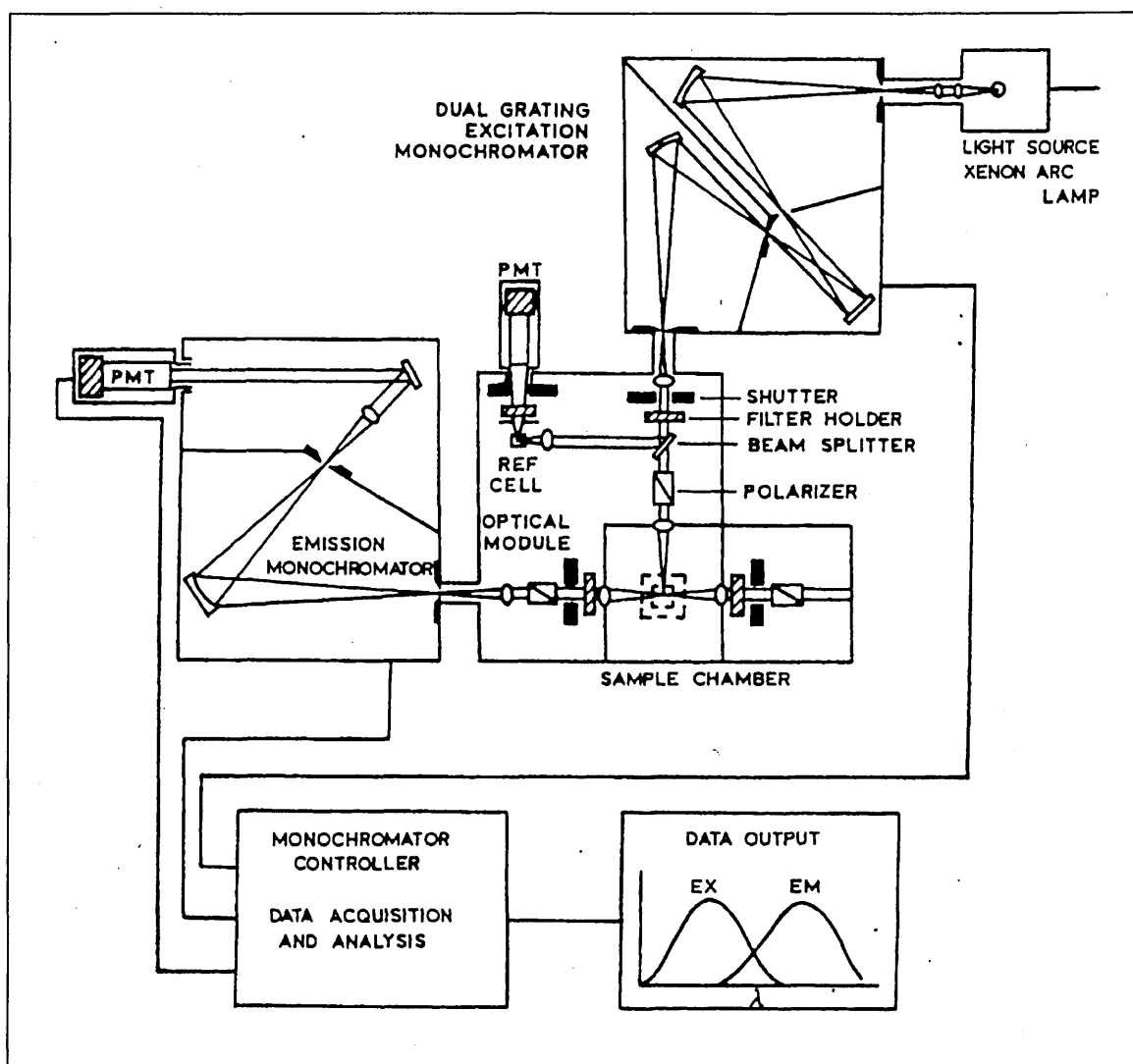


Figure 1. 15 Schematic diagram of a spectrofluorimeter. PMT stands for photomultiplier tube (33).

1.9 AIMS AND OBJECTIVES

Significant advances in the understanding of the biochemistry of selenium have been made in the last decade. A number of analytical techniques and methods have been applied to the determination of the element in various matrices. From the analytical methods and techniques reviewed it was found that fluorimetry, HGAAS, HG-ICP-AES and ICP-MS (with and without HG) are the most commonly employed for routine laboratory determination of Se, although the fluorimetric methods are becoming dated with the use of new reagents as PTQA. There is still the need to develop cheap, robust and reliable methods and the fluorescence technique is a desirable one. The aims and objectives of this study are:

- (i) To develop and evaluate fluorimetric methods for the determination of selenium based on the use of PTQA as the fluorimetric reagent.
- (ii) To critically evaluate the existing methods used for the synthesis of PTQA and to develop an alternative route which will provide greater yield.
- (iii) To develop a UV photoreduction of Se (VI) to Se (IV) in a basic medium.
- (iv) To optimise the conditions for sample pretreatment, separation and detection for the study of selenium species.
- (v) To validate and apply the methods developed to the study of selenium distribution in selenium-containing human supplements using ICP-MS detection.

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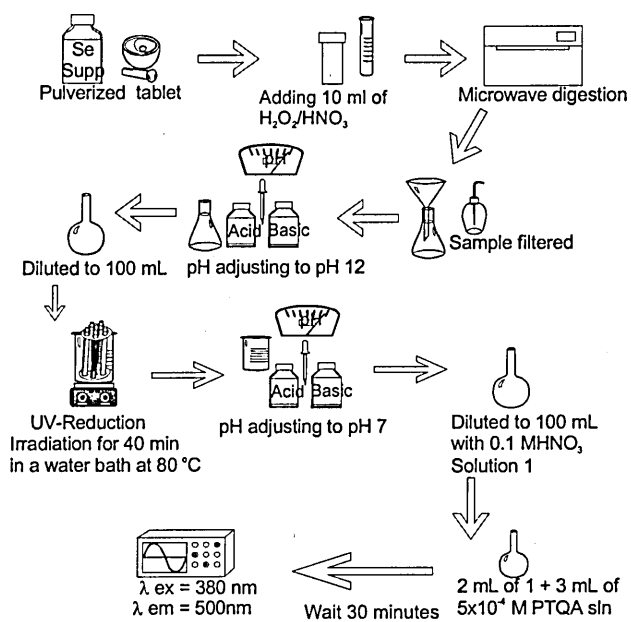
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Chapter 2:

Experimental



2.1 APPARATUS AND INSTRUMENTS

2.1.1 Balances

A Mettler Toledo (Switzerland) AB54 ($d = 0.1 \text{ mg}$) and a Mettler Toledo PB303 balance ($d = 1 \text{ mg}$) were used.

2.1.2 pH meter

A Model 7 Corning (Fisher Scientific, Loughborough, UK) pH meter was used.

2.1.3 Heaters and vacuum pump

The following equipment supplied by Fisher Scientific, Loughborough, UK was used: Electromantle MX, Thermodyne stirring hot plate, Challenge MH 3371 heater, JAVAC high vacuum pump, Vacuubrand vacuum pump, Buchi Rotavapor R-200. Melting points were taken using model SMP3 Stuart Scientific melting point apparatus.

2.1.4 Microwave digestion

A Milestone model MLS-1200 Mega microwave system supplied by Scientific & Medical Products Ltd. (Manchester, UK) was used for the acid digestion of the vitamin supplements. The microwave program is shown in Table 2. 1.

2.1.5 Photoreactor

A home-made photo-reactor composed of a Heraeus TNN 15/32 low pressure mercury vapour lamp, emission wavelength 254 nm, and dimensions of 17 cm long and 2.2 cm o.d. was used as the irradiation source. The batch and on-line configurations in which the

Table 2. 1 Microwave program.

Step	Power (W)	Time (minutes)
1	250	5
2	0	15
3	600	10
4	ventilation	10

lamp was used for the photoreduction of Se (VI) to Se (IV) are shown in Figures 2.1 and 2.2, respectively.

In the batch configuration, the Teflon frame, in which irradiation was carried out, consisted of two circular plates (17 cm diameter x 1 cm thickness), separated by three rods held in place by Teflon screws. A hole to hold the mercury lamp was drilled through the centre of the top plate. Five holes, each of diameter 2.2 cm, to take the quartz sample digestion vessels were drilled at equidistant points from the centre. A smaller hole to hold the thermometer was drilled closer to the lamp but was made such that radiation from the lamp was not shielded from a sample position. In use, the frame was inserted into a water bath, which is composed of a 5 litre steel beaker (Fisher Scientific, Loughborough, UK) placed on a hot plate (Fisher Scientific, Loughborough, UK) and covered with aluminium foil to increase light intensity and to protect the operator from exposure to damaging UV irradiation.

In the on-line version, no holes were drilled for the sample holders, but instead a length of Teflon tubing (0.5 mm i.d.) was wrapped around the Teflon frame.

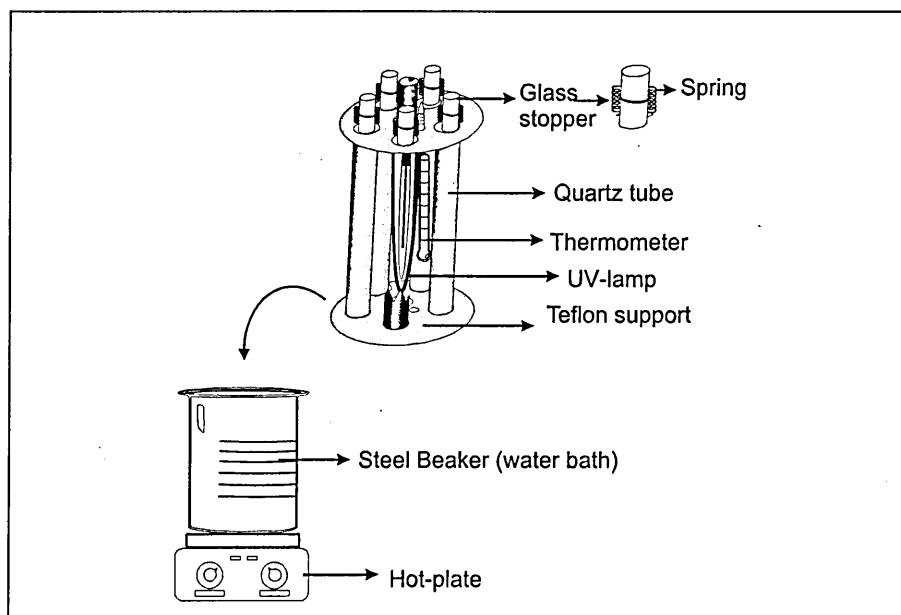


Figure 2. 1 Schematic diagram of the batch photoreactor.

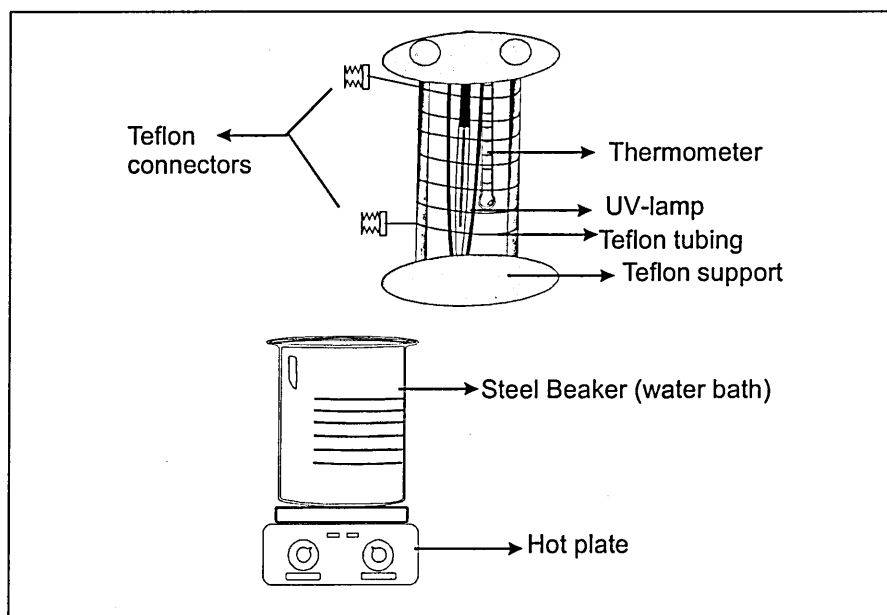


Figure 2. 2 Schematic diagram of the on-line photoreactor system.

2.1.6 Spectrofluorimeters

The following spectrofluorimeters were used: HITACHI model 1010 (Hitachi Instruments, Slough, UK), Perkin Elmer model LC-240 (PE Instruments, Beaconsfield, UK) and a Jasco model FP-920 (Jasco Limited, Great Dunmow, UK).

2.1.7 Inductively coupled plasma-mass spectrometer

The inductively coupled plasma-mass spectrometer (ICP-MS) used was a Hewlett Packard (HP) 4500 (Hewlett Packard, Yokogawa Corporation, Japan) fitted with a Scott double pass spray chamber and a Babington nebuliser. The operating conditions are shown in Table 2. 2.

Table 2. 2 Operation conditions for ICP-MS.

Condition	Regular ICP method	Hydride generation method
Forward power	1250 W	950 W
Plasma gas flow	12.0 L min ⁻¹	12.0 L min ⁻¹
Auxiliary gas flow	1.28 L min ⁻¹	1.20 L min ⁻¹
nebuliser gas flow	1.19 L min ⁻¹	1.19 L min ⁻¹
Blend gas	Argon	Argon
Sample depth	4.8 mm	4.5 mm
Monitoring mass (T.R.A.)	m/z 82	m/z 77, 82
Integration time/mass	3 s	3 s

2.1.8 The flow-injection (FI) system

Figure 2. 3 shows the schematic diagram of the developed flow injection (FI) system. A four channel peristaltic pump (Minipuls 3 Gilson, Anachem Ltd., Luton ,UK) fitted with Viton rubber tubes 0.80, 1.00 and 1.60 mm i.d (Altec, Bude, UK) was used to pump solutions through 0.5 and 0.8 mm i.d. Teflon tubing connecting the mixing coils and photoreactor. Samples were injected into a Rheodyne low pressure injection valve (Supelco, Poole, UK). A switching valve (Rheodyne, supplied by Supelco, Poole,UK) was used to control the flow of the samples and reagents through the FI system. The on-line photoreactor described previously was connected to the FI system for the photo-reduction of Se (VI).

2. 2 REAGENTS AND CHEMICALS

The following AnalaR grade reagents were used: nitric acid (HNO_3) 69%, hydrogen peroxide 30% (H_2O_2), sulphuric acid (H_2SO_4) 98%, hydrochloric acid (HCl) 37%, hydrobromic acid (HBr) 47 %, methanol (CH_3OH), ethanol ($\text{CH}_3\text{CH}_2\text{OH}$), isopropanol ($(\text{CH}_3)_2\text{CHOH}$), dichloromethane (methylene chloride, CH_2Cl_2), toluene (C_7H_9), n-hexane (C_6H_{14}), cyclohexane (C_6H_{12}), acetone and acetic acid (CH_3COOH) all supplied by Merck, BDH (Poole, UK). Petroleum spirit (40-60 °C), and ethanol analytical grade was supplied by Prime Chemicals (Rotherham, UK); Quinaldine 95 +% was obtained from Aldrich, Gillingham, UK.

Sodium hydroxide, potassium hydroxide pellets, sodium selenite 99 % (Na_2SeO_3), sodium carbonate anhydrous 99.9 % (Na_2CO_3), potassium peroxodisulfate 99.0%, potassium iodide 99.5 % were purchased from BDH Merck (Poole, Dorset, UK). Potassium bromide 99.5 % was supplied by Prolabo. Sodium selenate (Na_2SeO_4), and seleno-DL-methionine ($\text{C}_5\text{H}_{11}\text{NO}_2\text{Se}$) were from Fluka. 2- amino-pyridine 99 %, sulphur powder 99.98 %, phosphorus pentasulphide 99% seleno-D,L-methionine were purchased from Aldrich Co. Ltd (Poole, Dorset, UK). seleno-DL-cystine ($\text{C}_6\text{H}_{12}\text{N}_2\text{O}_4\text{Se}_2$) from Sigma. A 1000 mg L^{-1} solution of indium (In), and selenium (Se) supplied by Merck, BDH (Poole, UK). Two certified water reference material solutions, 51.2 and 23.2 from National Water Research Institute Environment Canada, were used for the validation of the ICP-MS method (Table 2. 3). The nutritional supplements were purchased from a local health shop. The composition of the nutritional supplements are shown in Table 2. 4.

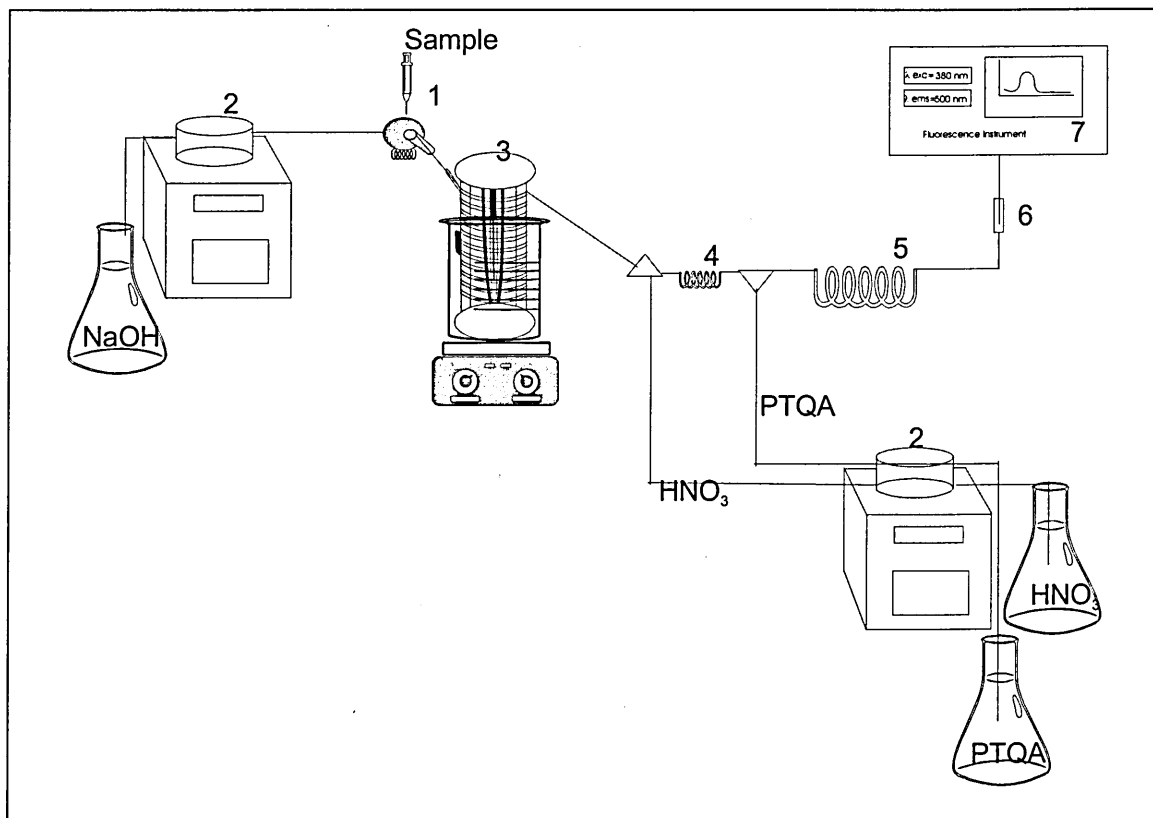


Figure 2. 3 Schematic diagram of the developed flow injection system coupled to the fluorimetric detector for the determination of Se (IV) and Se (VI). 1 injection valve, 2 peristaltic pump, 3 photoreactor, 4 and 5 mixing coils, 6 on-line filter, 7 fluorimeter.

Separate 1000 mg L⁻¹ Se (IV) and Se (VI) standards were prepared by dissolving 110.6 and 122.1 mg of AnalaR grade sodium selenite and sodium selenate, respectively, in 50 mL deionized water. Working standards were prepared daily by appropriate dilution in 1 M HNO₃.

Solutions of 2.5 and 5.0 M of NaOH were prepared daily. Solution of 5×10⁻⁴ M PTQA was prepared daily by dissolving 33.1 mg of PTQA in 250 mL of isopropanol.

Table 2. 3 Certified values for trace elements in TMDA 51.2 and 23.2.

Element	TMDA 51.2 ($\mu\text{g L}^{-1}$)	TMDA 23.2 ($\mu\text{g L}^{-1}$)
Aluminium	96.0 \pm 19.0	97.0 \pm 13.0
Antimony	12.5 \pm 2.7	2.6 \pm 0.63
Arsenic	15.3 \pm 3.4	8.5 \pm 1.6
Barium	73.3 \pm 6.1	14.6 \pm 3.0
Beryllium	9.1 \pm 1.5	1.6 \pm 0.38
Cadmium	25.1 \pm 2.5	2.6 \pm 0.65
Chromium	62.5 \pm 6.6	6.5 \pm 1.4
Cobalt	71.9 \pm 6.3	7.6 \pm 1.5
Copper	91.0 \pm 10.2	9.6 \pm 1.9
Iron	111.0 \pm 25.8	12.6 \pm 3.5
Lead	72.9 \pm 10.6	3.8 \pm 1.0
Manganese	82.0 \pm 10.2	8.4 \pm 1.3
Molybdenum	58.5 \pm 7.4	5.0 \pm 1.5
Nickel	66.7 \pm 7.4	5.3 \pm 1.3
Selenium	12.0 \pm 7.4	4.2 \pm 1.4
Strontium	121.0 \pm 12	59.5 \pm 4.4
Thallium	20.0 \pm 3.2	2.1 \pm 0.74
Uranium	29.3 \pm 7.3	10.8 \pm 3.2
Vanadium	47.7 \pm 7.7	
Zinc	106.0 \pm 15	

Table 2. 4 Selenium content and mineral composition of the vitamin supplements analysed by the developed analytical methods.

Sample	Composition	Expected Se Concentration in 100 mL
A	Dicalcium phosphate, microcrystalline cellulose, L-Selenomethionine (Se 200 µg), vegetable sourced magnesium stearate, vegetable sourced stearic acid.	Se 2000 µg L ⁻¹
B	E 67 mg, B1 5 mg, B2 5 mg, B3 10 mg, B6 50 mg, Pantothenic Acid 10 mg, Mg 175 mg, Mn 2 mg, B 1 µg, Se 25 µg, Cr 25 µg	Mg 1750 µg L ⁻¹ , Mn 20 µg L ⁻¹ , B 10 µg L ⁻¹ , Se 250 µg L ⁻¹ , Cr 250 µg L ⁻¹
C	A 1000 µg, C 200 mg, E 83.9 mg, Se 25 µg	Se 250 µg L ⁻¹
D	A 450 µg, E 30 µg, C 90 mg, Selenium 100 µg Calcium phosphate, microcrystalline cellulose, potato starch, silicon dioxide, magnesium stearate, gum acacia, iron oxides (natural colours)	Se 1000 µg L ⁻¹
E	Dicalcium phosphate, microcrystalline cellulose, brewers'yeast, calcium sulphate, selenium yeast (source of selenium) Se 50 µg (all natural yeast), vegetable sourced stearic acid, colloidal silicon dioxide.	Se 500 µg L ⁻¹
F	E 67 mg, B1 5 mg, B2 5 mg, B3 10 mg, B6 50 mg, Pantothenic Acid 10 mg, Mg 175 mg, Mn 2 mg, B 1 mg, Se 25 µg, Cr 25 µg	Mg 1750 µg L ⁻¹ , Mn 20 µg L ⁻¹ , B 10 µg L ⁻¹ , Se 250 µg L ⁻¹ , Cr 250 µg L ⁻¹

2.3 PROCEDURE

2.3.1 2- (α-Pyridyl) thioquinaldinamide (PTQA) synthesis

In the course of this study, a number of methods for PTQA synthesis were investigated.

2.3.1.1 PTQA synthesis after Ahmed et al (1)

A mixture of quinaldine, sulphur and 2-amino-pyridine in the molar ratio of 0.007:1.5:2.0 was transferred into a round bottom flask and heated at about 150 ° C for 6 hours using a hot plate. The reaction mixture was transferred to a beaker and

left to cool overnight. The solid product was pulverized and washed five times with petroleum ether. The yellow crystals were recrystallised in ethanol.

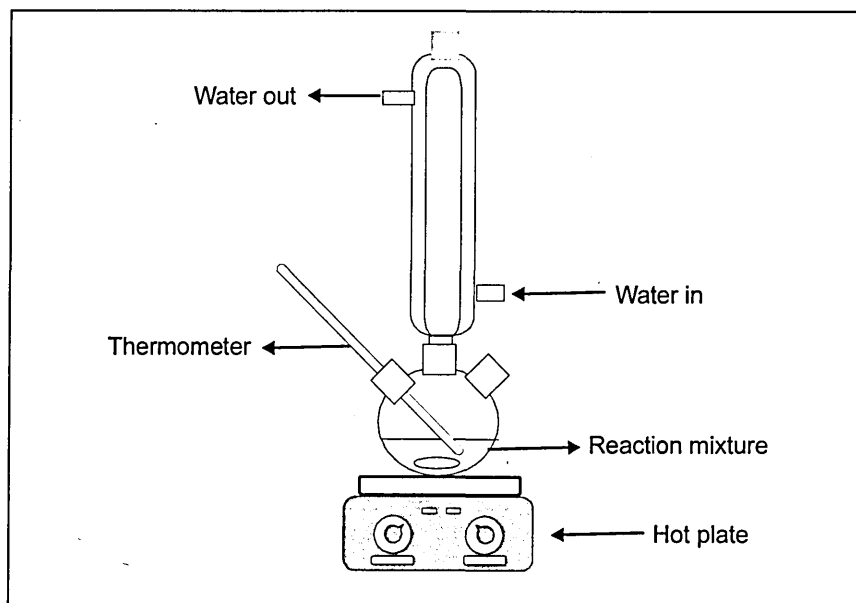


Figure 2. 4 Refluxing system for PTQA reaction.

2.3.1.2 PTQA synthesis using phosphorus pentasulphide (P_4S_{10})

In 20 mL of toluene a mixture of quinaldine carboxylic acid, phosphorus pentasulphide and 2-amino-pyridine in a molar ratio of 1.0:1.5:2.0 was stirred in a round bottom flask at 100 °C, for 12 hours.

2.3.1.3 PTQA synthesis by active methyl condensation, with 2-amino-pyridine in presence of sulphur

This method of synthesis was based on combination of various elements from methods of Ahmed (1), Porter (2), Saikachi and Hisano (3), and Mansfield (4). The reaction conditions, and the chemical proportions used are shown in Table 2. 5.

Table 2. 5 Reaction conditions used in the synthesis of PTQA.

Reagent Molar Ratio Quinaldine:sulphur:2-amino-pyridine	Reaction Time (hours)	Temperature (°C)
0.1:0.15:0.2	12	140 ☼
0.1:0.15:0.2	3, 6, 12, 24	150 ☼
0.1:0.15:0.2	12	160 *

☼ Hot plate, * Oil bath.

Simple crystallisation, vacuum distillation, with crystallisation, as well as column separation were used for purification. The procedures are described in **section 2.3.2.**

2.3.1.4 Patent DNEPR chem techn Ins. 055R 1985 112 (5)

A mixture of quinaldine, 2-amino-pyridine, sulphur and sodium sulphide in mole amounts of 0.1, 0.15, 0.30, and 0.008 respectively was refluxed with stirring at 150-160 ° C for 12 hours in a round bottom flask. After the reaction, the excess amine and quinaldine were removed by vacuum distillation and the final mixture was crystallised in ethanol. Reactions were carried out at 3, 6, 12, and 24 hours in order to select the optimum reaction time. The reaction mixture was purified using column separation as described in **section 2.3.2.3.**

2.3.2 PTQA purification

2.3.2.1 Crystallisation

The reaction mixture was washed with 3 portions of 100 mL of hot petroleum ether. After cooling the yellow solid was filtered and re-crystallised in ethanol. The melting point of the product was then taken.

2.3.2.2 Vacuum distillation and crystallisation

To remove the unreacted quinaldine and 2-amino-pyridine, the reaction mixture was vacuum distilled. After distillation the remaining solid was washed with petroleum ether and the solid was filtered by suction. The yellow solid was recrystallised in ethanol.

2.3.2.3 Glass column separation

The separation column was prepared with 1 kg silica gel. A slurry, prepared from the reaction mixture and silica gel was applied to the top of the column. Chloroform was used to elute the fractions from the column. The PTQA-containing fraction was evaporated to dryness and recrystallised in 50:50 petroleum ether: dichloromethane

2.3.3 Sample pretreatment

2.3.3.1 Total digestion

Pulverised selenium-containing supplement tablet or a weight between 100-200 mg of seleno-DL-methionine or seleno-DL-cystine was transferred into the Teflon vessel. 10 mL of the acid digestion solution was added. The digestion solutions investigated include: 1:1 $\text{HNO}_3\text{:H}_2\text{O}_2$, 1:1 $\text{H}_2\text{SO}_4\text{:H}_2\text{O}_2$, 1:1:1 $\text{H}_2\text{SO}_4\text{:HNO}_3\text{:H}_2\text{O}_2$ and H_2O_2 . Ten samples held in a carousel were digested simultaneously using the program shown in Table 2. 1. The digests were made up to 100 mL with deionised water.

2.3.3.2 Selenium extraction

Mechanical leaching

Supplement tablets were pulverised and placed into a 125 mL flask with 50 mL of the extraction solvent or mixture. The extraction solutions used included: 2.5 M NaOH, 5 M NaOH, 2.5 M HNO_3 , 1:1 methanol:water, water, and $\text{Na}_2\text{CO}_3\text{:NaHCO}_3$ 1.7:1.8, respectively. Samples were shaken for 12 or 24 hours. The extract samples were filtered and the filtrate diluted to 100 mL with deionised water.

Ultrasonic extraction

A pulverised tablet was placed in a 125 mL flask with 20 mL of the extraction solution. The flask was placed into an ultrasonic bath for 12 or 24 hours, after which the sample was filtered and diluted to 100 mL with deionised water.

2.3.4 Fluorescence detection

2.3.4.1 Off-line photoreduction

25 mL from the total digest (see section 2.3.3.1) was neutralised with 6M NaOH solution and diluted to 50 mL with 5 M NaOH. A 25 mL aliquot of the basic solution or of the Se (VI) standard solution was transferred into the quartz digestion tube. The sample was irradiated for 40 min at 80 °C. After irradiation, the pH of the solution was adjusted to 7 with a volume of between 1 or 0.5 mL concentrated HNO_3 .

Table 2. 6 summarises the parameters optimised in developing the fluorimetric method for the determination of selenium. To obtain the fluorescence signal, 2 mL of sample is prepared in 0.1 M HNO₃, and mixed with 3 mL of the PTQA solution. The fluorescence intensity of the resulting solution was measured.

Table 2. 6 Parameters studied for the PTQA-Se (IV) reaction.

Condition studied	
Acid type and concentration	HNO ₃ , H ₂ SO ₄ , H ₃ PO ₄ , HCl, acetic acid, from 0.01 to 1 M
PTQA solvent	Methanol, ethanol and isopropanol
PTQA concentration	5×10^{-6} to 2×10^{-3} M
Reaction time	5 to 90 minutes with interval of 5 minutes

2.3.4.2 On-line photoreduction

In order to optimise the conditions for the determination of Se (IV) and Se (VI), 1.00 mL of the separate standards (20 –500 µg L⁻¹) was injected into the carrier stream at a flow rate of 0.40 mL min⁻¹. For the determination of Se (VI), the sample was injected into the 5 M NaOH stream and pumped into the photoreactor, where the flow was stopped for 40 minutes to allow the complete reduction of Se (VI) to Se (IV). After the irradiation, the sample stream was mixed with a stream of 2.5 M HNO₃, with a flow rate of 0.92 mL min⁻¹. In order to reduce the pH, the resulting stream was mixed with the PTQA solution and pumped into the fluorimeter. Simultaneously a second sample was injected by-passing the photoreactor for the determination of Se (IV). Figure 2. 5 shows a diagram of the on-line system for the simultaneous determination of Se (IV) and Se (VI). This system is a modification from the one shown in Figure 2. 3. A by-pass valve is include in this set-up. In the period in which selenate is reduced, five samples of Se (IV) can be determined. The optimised excitation and emission wavelengths were 380 and 500 nm, respectively. Peak area was used for signal evaluation. All measurements were performed in triplicate.

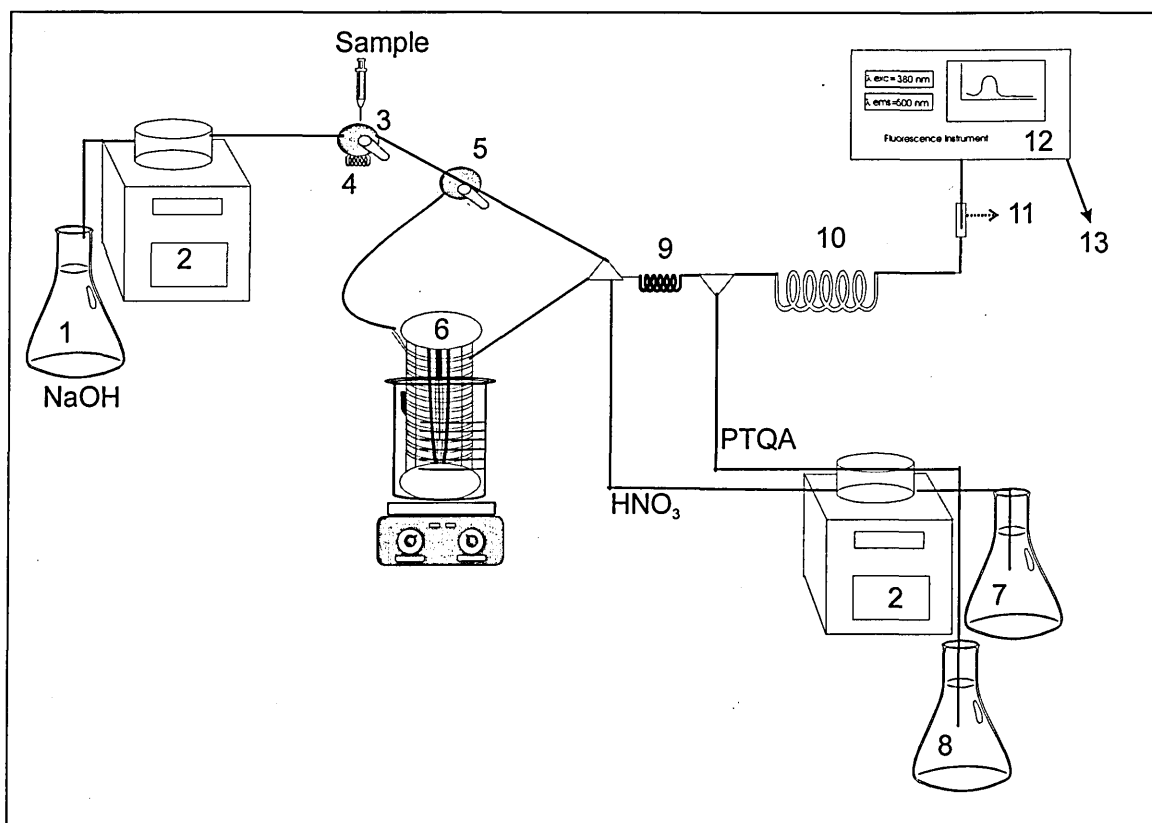


Figure 2. 5 System for Se (IV) and Se (VI) determination with a continuous flow using a by-pass valve. 1 5 M NaOH; 2 peristaltic pump; 3 injected valve; 4 sample loop (1000 μ L); 5 by-pass valve; 6 photoreactor; 7 2.5 M HNO_3 ; 8 5×10^{-4} M PTQA; 9 and 10 mixing coil; 11 on-line filter; 12 fluorimeter; 13 waste.

2.3.5 ICP-MS methods

2.3.5.1 Total selenium

Indium at a concentration of $10 \mu\text{g L}^{-1}$ was added to the standards and samples as an internal standard. Also sodium and calcium at a concentration of 50 mg L^{-1} were added to the standards (matrix-matched).

2.3.5.2 Selenium speciation using HPLC-UV-HG-ICP-MS

The instrumental conditions were those previously optimised by Mawhinney (6). 50 μL of sample were injected into the system (Figure 2. 6) for its separation. The column eluate was mixed with 5 M NaOH, 0.5 mL min^{-1} , and the mixture was pumped to the photoreactor to reduce Se (VI) to Se (IV). After UV-irradiation the stream was mixed with 8.4 M HCl and 1% NaBH_4 in 0.1 M NaOH to generate the corresponding hydride, which was separated by the gas-liquid separator unit and the

gaseous hydride was transferred in an argon stream to the ICP-MS instrument for selenium detection.

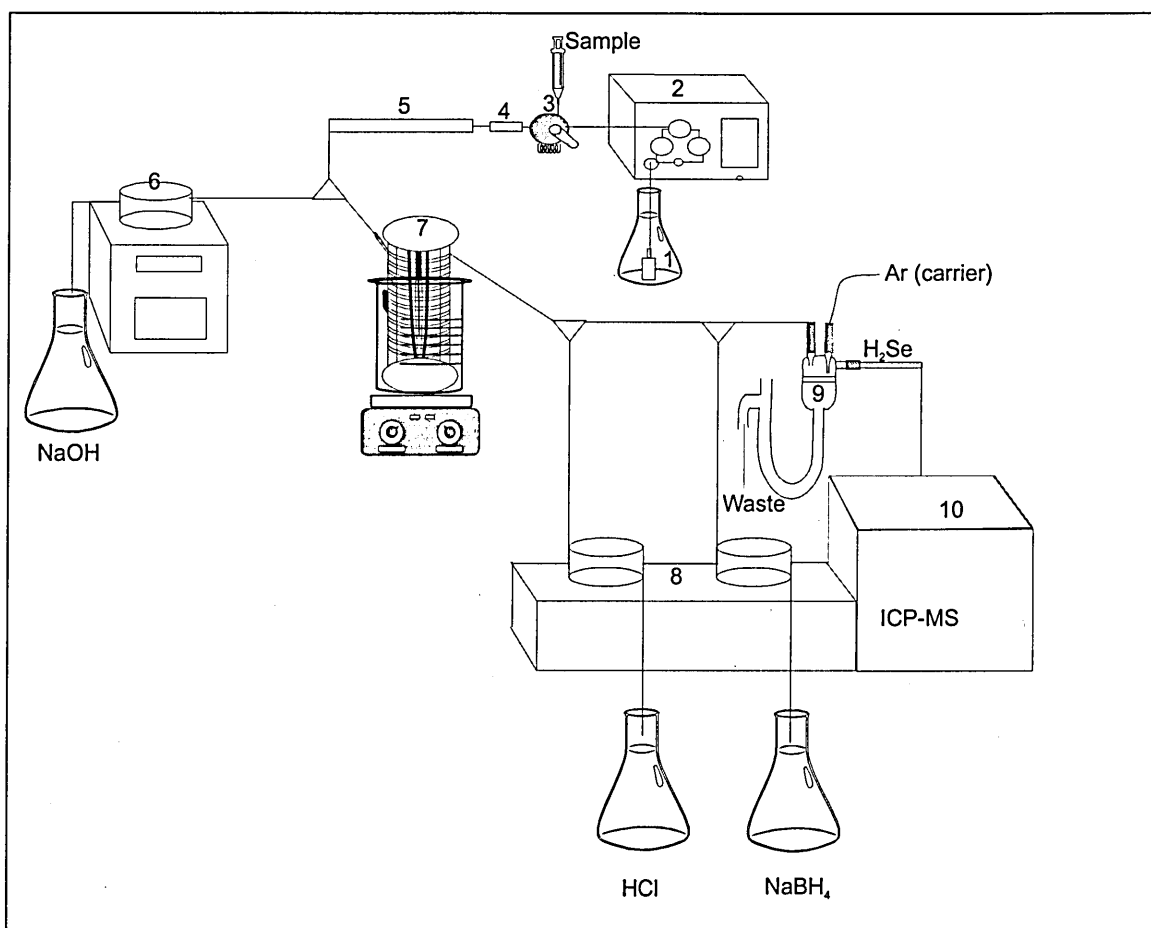


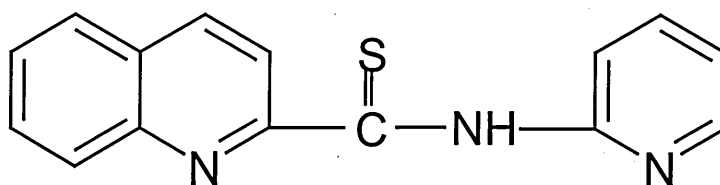
Figure 2. 6 Ion-HPLC-HG-ICP-MS system. 1 mobile phase $\text{Na}_2\text{CO}_3/\text{NaHCO}_3$ 1.7/1.8, 2 HPLC pump, 3 injection valve (50 μL), 4 Ion Pac AG9-SC 4 mm (10-32) P/N 43186 Dionex, 5 Ion Pac AS9-SC 4 mm p/n 43185 Dionex, 6 peristaltic pump, 7 photoreactor, 8 ICP-MS peristaltic pump, 9 gas-liquid separator, 10 ICP-MS instruments.

2.4 REFERENCES

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- 4 Mansfield, R.; Preparation of some N-t-Alkylthiopicolinamides, *Journal Organic Chemistry*; **24**; 1111-1114; 1959.
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Chapter 3:

Synthesis and Purification of PTQA



PTQA



3.1 INTRODUCTION

N- (α -Pyridyl)-2- thioquinaldinamide (PTQA) [6], a quinolinethioamide compound, was first used for the photometric determination of copper (II) by Chakrabarti (1). Pal et al. (2) introduced PTQA as a fluorimetric agent. The compound used in the first two applications was synthesised by the method described by Porter (3). Subsequently PTQA has been used as a fluorimetric reagent for the determination of Se (IV) (2-5), Cr (VI) (5, 6), Mn (VII) (5, 7), As (III) (8), Ru (III) (9), and for V (IV and V) (10). In the study reported by Porter, seven thioamide compounds were produced from the reaction of α or γ - picoline (α or γ - methylpyridine) with an amine in the presence of sulphur. This is a condensation reaction in which a methyl compound, picoline reacts with an aromatic primary amine in the presence of sulphur to produce a thioamide. In the Porter reaction, 3 moles, 1 mole and 2.5 moles of sulphur, α -picoline [1] and aniline [2], respectively were mixed at 160 °C, for 12 hours in a heated oil bath. Unreacted α -picoline and aniline were removed by vacuum distillation. The residue remaining was dissolved in ethanol, and the ethanolic solution was distilled to obtain the final product, which was recrystallised with a 1:1 n-hexane/benzene mixture. The reported yield was 63 %.

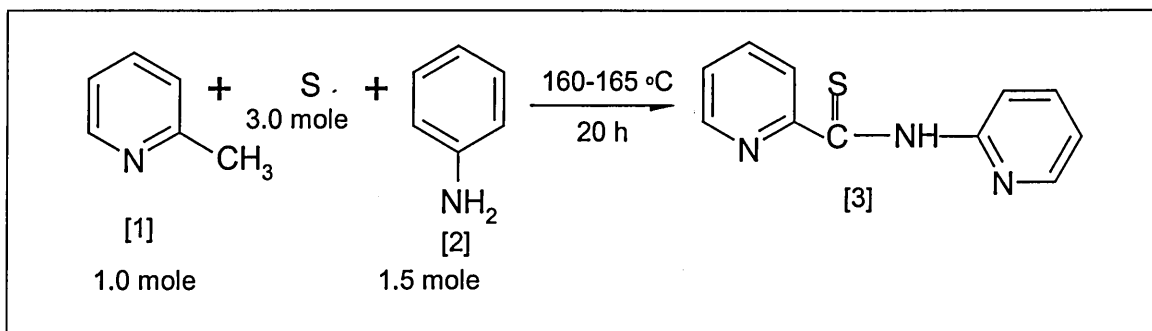


Figure 3. 1 Synthesis of PTQA homologues after Porter (3).

Recently, Ahmed et al. (4) described a modification of the Porter method for the synthesis of PTQA. A mixture of 0.007 mole of quinaldine (2- methylquinoline) [4] with 1.5 mole of sulphur and 2.0 mole of 2-amino-pyridine [5] was heated at 145-155 °C for 6 hours, and the product was left to cool over night. PTQA was recrystallised from the mixture using ethanol. No indication of the yield obtained was given. It is noteworthy that the amount of the other reactants reagents were in 200-fold excess of the amount of quinaldine. Such proportions are uncommon in organic synthesis.

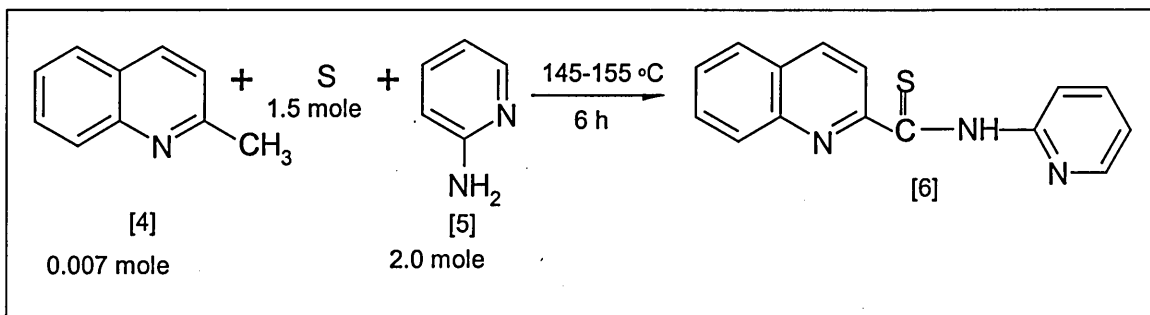


Figure 3. 2 Synthesis of PTQA after Ahmed et al. (4).

Saikachi and Hisano (11) as well as Ahmed (4) described the reaction of quinaldine and aromatic primary amines in the presence of sulphur. The differences between the Ahmed and Porter methods are in the use of aniline instead of 2-amino-pyridine, and the proportion of reactants. The mole proportion used by Ahmed was 0.025: 0.038: 0.063 for quinaldine, amine and sulphur, respectively. The reaction mixture was refluxed for 20 hr at 160-165 °C. Unreacted reagents were removed by vacuum distillation. The product was extracted with hot 10 % NaOH. The orange crystals formed after cooling of the extract were recrystallised in ethanol. As shown in Figure 3. 3, a minor product [9] was obtained in a poor yield.

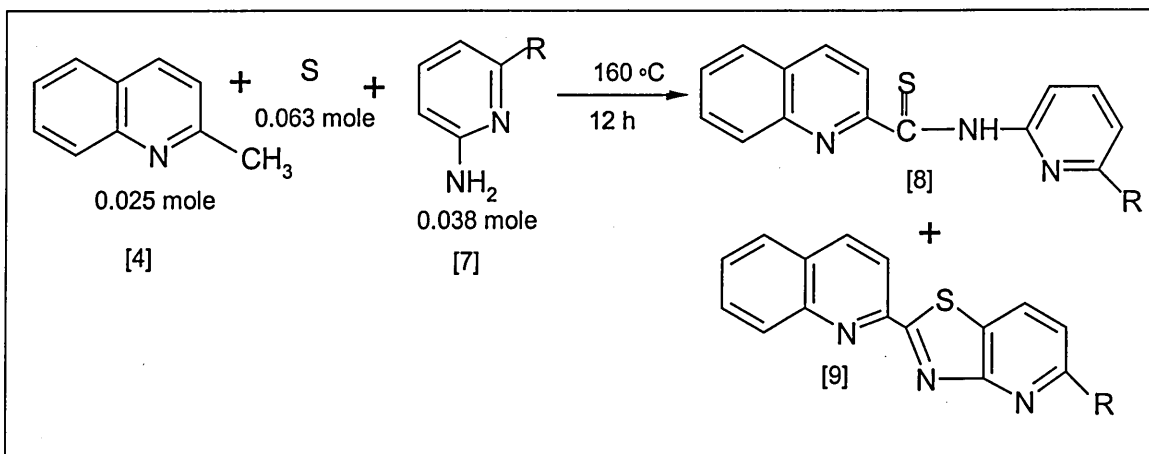


Figure 3. 3 Reaction scheme of the synthesis developed by Saikachi and Hisano (11).

Subsequent work was reported by Mansfield (12) on the synthesis of N-t-alkylthiopicolinamides (alkylthiomethylpyridineamides) in which the molar proportion of picoline, amine, and elemental sulphur were studied. Good yields were obtained when excess amounts of pyridine and sulphur were used, and the reactants were refluxed at temperatures between 150 and 200 °C for 24 hours.

A similar synthesis reported in a Russian patent (13) recommended the use of sodium sulphide as a catalyst. The reagents, which include the active methyl compound, α -picoline, the primary amine, sulphur and Na_2S are heated between 150-160 °C for 12 hours. Unreacted amine and α -picoline were recovered by

vacuum distillation. The product was washed with hot petroleum ether, excess sulphur was filtered from the solution and the final product was distilled. A yield of 75.1 % was obtained after recrystallisation in 2-propanol. The introduction of sodium sulphide shortened reaction time and improved the yield.

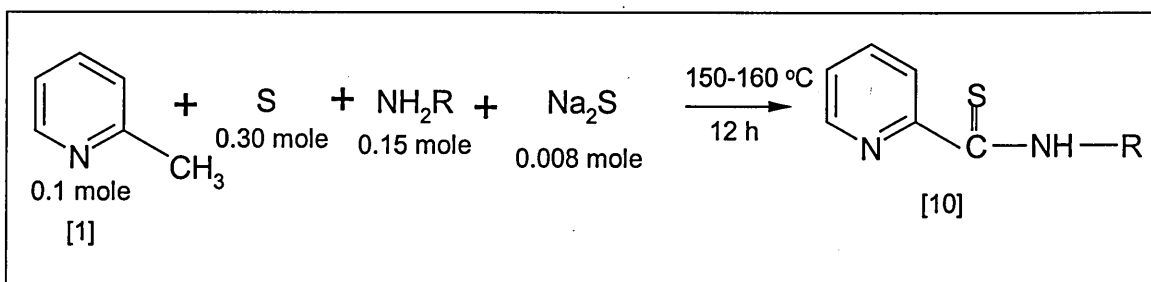


Figure 3. 4 Reaction scheme of the synthesis of a thioamide compound catalysed by Na₂S as reported in patent DNEPR Chem Techn Ins (13).

Conversion of a carbonyl group to thiocarbonyl is an alternative method for the active methyl condensation reaction. Using this approach, Steliou and Mrani (14) described a new method for the conversion of carbonyl units into their corresponding thiocarbonyl analogues. Boron sulphide is used as the sulphur source, which reacts with the carbonyl group to form the corresponding thione as shown in Figure 3. 5. The organometallic sulphide transports sulphur to the reaction medium. Using this technique, aldehydes, ketones, lactones, and lactams can be readily converted into their corresponding thioanalogues to give high yields of products.

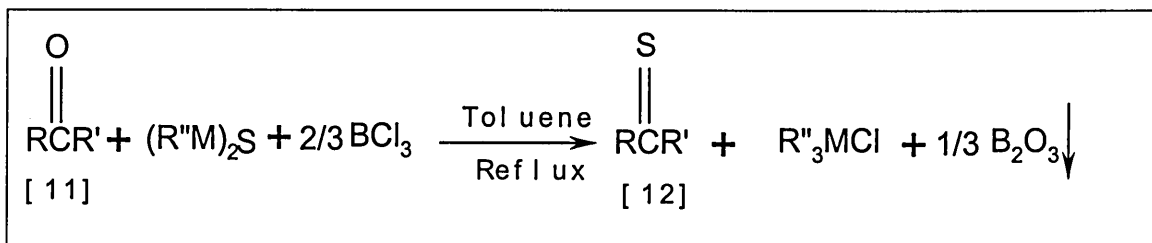


Figure 3. 5 Reaction proposed by Steliou and Mrani (14).

More recently, McGregor and Sherrington (15) have reviewed the synthetic routes to thioketones and thioaldehydes. Five methods to convert carbonyls into thiocarbonyls were examined, as well as five routes to obtain thiocarbonyl compounds from non-carbonyl precursors being discussed. The methods are summarised in Table 3. 1. In converting carbonyls to thiocarbonyls, hydrogen sulphide in acidic medium, reversibly protonates the carbonyl group, which is attacked by H₂S, and finally H₂O is eliminated to produce the thiocarbonyl compound. This method is used for both aldehydes and ketones. The application of this approach to aldehydes is less straightforward and it does not produce free thioaldehyde but the final product is a

cyclic adduct. One disadvantage of using these methods is the difficulty associated with the use of hydrogen sulphide. However, a high yield is obtained and hence the use of hydrogen sulphide is a minor inconvenience.

Table 3. 1 Summary of the methods reviewed by McGregor and Sherrington for the conversion of carbonyl groups into thiocarbonyls (15).

Reaction	Advantages/Disadvantages
Carbonyl conversion routes	
Hydrogen Sulphide in acid ($\text{H}_2\text{S}/\text{HCl}$)	Free thioaldehyde not produced.
Phosphorus and sulphur compounds	Low reagent low solubility but good yield, and no organic by-products.
Silicon- and tin-based reagent	Produce stable aldehydes which are isolated as Diels-Alder cycloadducts
Hydrazones with 1 S_2Cl_2 or S_2Cl_2 /base or 2 Ph_3PBr_2 /base and S_8	Stable thioketones
From non-carbonyl precursors	
Elimination of HX	Good yield with few by-products
Use of Xanthates	Variable yield from poor to good. Purification required to by-products.
Photolysis of thioacetophenones	Unstable aldehydes usually trapped in situ as Diels-Alder cycloadducts
Thermolysis of 3,3,5,5- tetraphenol 1-2-4-trithiolane (27)	Some thioaldehydes are trapped as Diels-Alder cycloadducts

The second route for the production of thiocarbonyl is the use of phosphorus-sulphur-based reagents. Different phosphorus and sulphur compounds have been described for the conversion of ketones into thioketones or related thiocarbonyls but phosphorus pentasulphide is the most commonly used. The main problem with this route is the insolubility of phosphorus pentasulphide in most organic solvents. However, phosphorus pentasulphide is soluble in pyridine. The advantages of this route include good yield, no organic by-products, and the application of simple purification procedures.

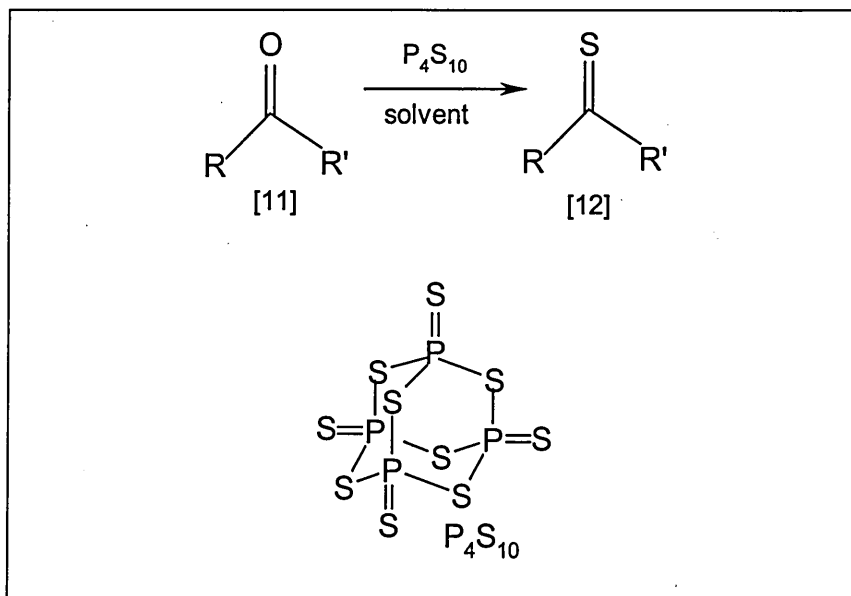


Figure 3. 6 Thionation with phosphorus pentasulphide (15).

The proposed third route is the use of silicon- or tin-based reagents. Initially SiS_2 was used to convert carbonyls into thiocarbonyls, but other silicon reagents have now become available. Bis (trimethylsilyl) sulphide in acetonitrile and in the presence of catalytic amounts of butyl lithium converts aldehydes into transient thioaldehydes, which are isolated in high yield as Diels-Alder cycloadducts.

The synthesis route via the formation of dithiolanes is exclusive to ketones. First stable thioaldehydes were synthesised via hydrazones, by reacting sulphur dichloride with triethylamine. Similar, products are obtained from the phosphorazone [14] as shown in Figure 3. 7.

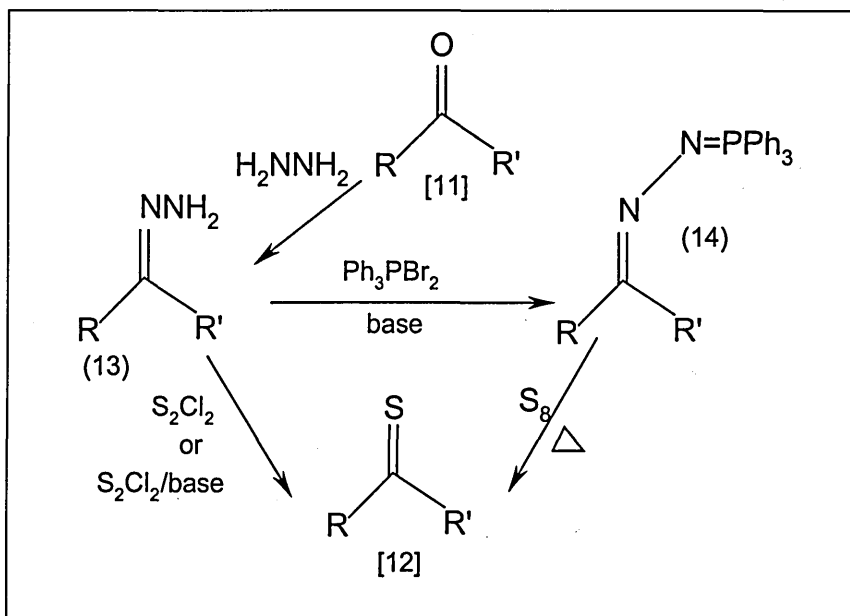


Figure 3. 7 Routes for the conversion of hydrazones into thiocarbonyls (15).

The synthesis of thiocarbonyl compounds from non-carbonyl precursors involves the elimination of HX. A large variety of thioaldehydes have been made by base-induced elimination of HX (Figure 3. 8), resulting in good yields and fewer by-products.

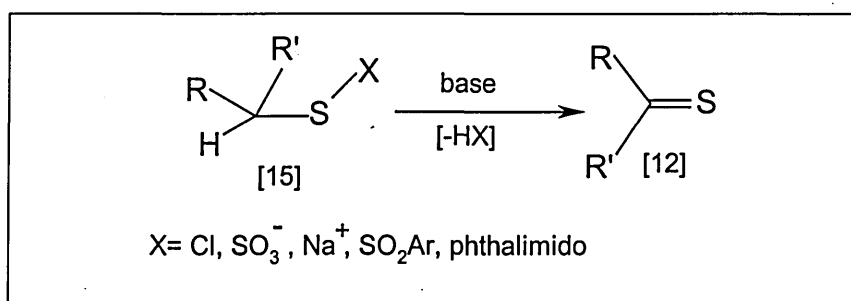


Figure 3. 8 Elimination of HX from sulphides (15).

Recently, gas phase dehydrocyanation has been reported for the production of thioaldehyde and thioformaldehyde as shown in Figure 3. 9.

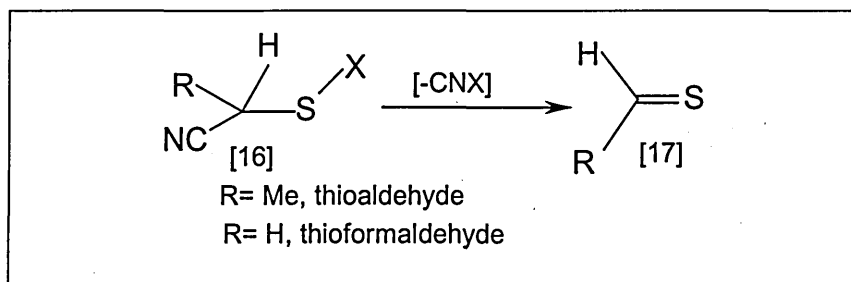


Figure 3. 9 Dehydrocyanation of cyanothiols (15).

Geminal dibromides [17] react with two equivalents of potassium O-ethyl xanthate (potassium O-ethyl dithiocarbonate, $C_3H_5KOS_2$) to give the thioketone or

thioaldehyde as shown in Figure 3. 10. The mechanism involves the possible displacement of bromide by one xanthate, then a second xanthate causes the elimination of another bromide to yield the final product. Varied yields and a number of by-products are obtained with this reaction.

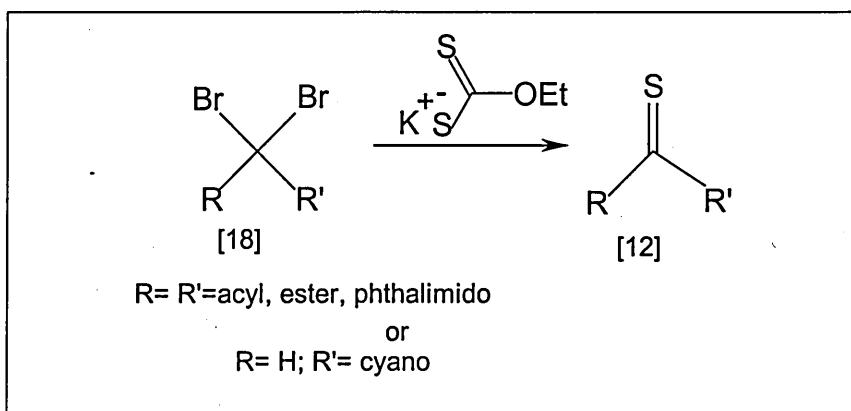


Figure 3. 10 Conversion of geminal dibromides into thiocarbonyls (15).

Photolysis of thioacetophenones has also been used for the synthesis of thiocarbonyl compounds. ω -Substituted thioacetophenones, synthesised from the reaction of thiolates with a halide, are cleaved in the presence of UV-light to produce thiocarbonyl compounds as shown in Figure 3. 11. Stable thioketones are produced by this method, but the thioaldehydes are not very stable and are trapped in-situ as Diels-Alder cycloadducts.

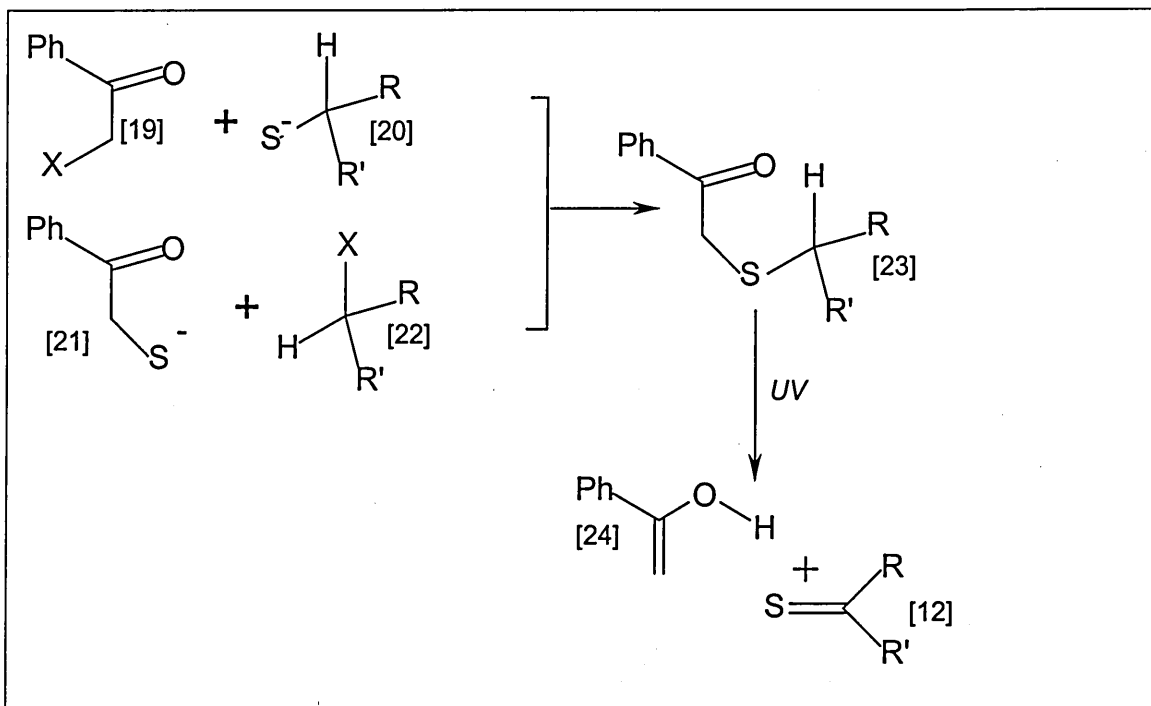


Figure 3. 11 Photolytic cleavage of thioacetophenones (15).

Syntheses based on the use of non-carbonyl compounds include the use of pyrolytic methods, the most common being the retro-Diels-Alder, as shown in Figure 3. 12.

Anthracene and cyclopentadiene adducts can thermally decompose to give thiocarbonyl compounds, which can react in situ with another substrate, such as cyclopentadiene or any other diene compound to produce a Diels-Alder product.

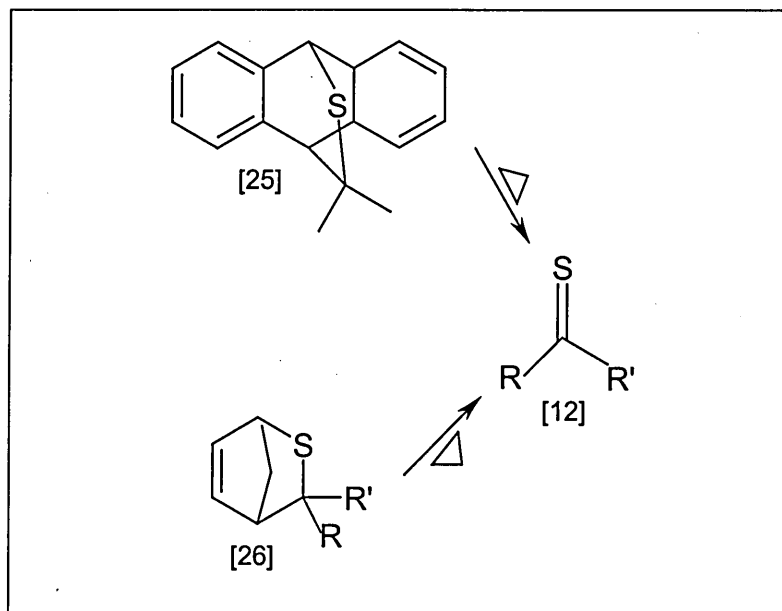


Figure 3. 12 Generation of thiocarbonyls by the retro-Diels-Alder (15).

A more unusual reaction is the thermolysis of 3,3,5,5-tetraphenyl-1,2,4-trithiolane, which decomposes above its melting point (124°C) to give a mixture of thiobenzophenone and ylid (28), which decomposes spontaneously to the thiocarbonyl and elemental sulphur as shown in Figure 3. 13.

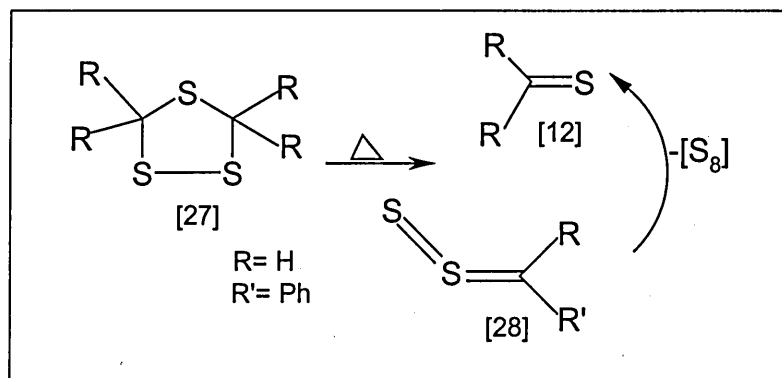


Figure 3. 13 Thermolysis of thiosulfinates (15).

Thiosulphinates decompose thermally to give sulphinic acid and thioaldehyde, which is trapped in situ as Diels-Alder cycloadducts, (see Figure 3. 14).

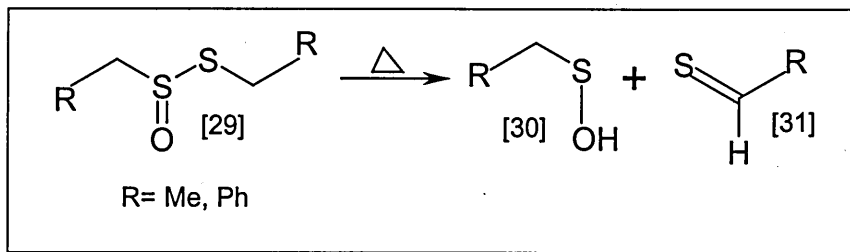


Figure 3. 14 Thermolysis of thiosulphonates (15).

Thioformaldehydes have been produced from 3,3,5,5-tetraphenyl-1,2,4-trithiolane [27] using flash vacuum pyrolysis (FVP).

From the methods reviewed by McGregor and Sherrington, the use of phosphorus pentasulphide for carbonyl conversion is the most attractive because of the high yield and the low number of by-products. Furthermore HX elimination, from a non-carbonyl precursor, is an alternative route for thiocarbonyl production, since this route results in good yields and fewer by-products.

In this chapter, PTQA synthesis and purification are optimised in a bid to develop a simple, efficient and cost effective method for the preparation of the compound. Initially the method reported by Ahmed was used, but modification based on the investigations by Porter (3), Saikachi and Hisano (11), and Mansfield (12) were made in order to obtain higher yields.

3.2 RESULTS AND DISCUSSION

3.2.1 Reaction and yield using Ahmed's method

Initially, the reaction was carried out using the molar proportion of 0.007, 1.5, 2.0 of quinaldine, sulphur and α -amino-pyridine, respectively as proposed in the original paper (4). After recrystallisation, the product yield was 16.8 %, and the melting point of the yellow needles was 151 °C which is comparable to 155 \pm 2 °C obtained by Ahmed et al. However, it was clear from the examination of the reaction equation, Figure 3. 2, that one mole of quinaldine will react with one mole of sulphur and one mole of 2-amino pyridine. It has been suggested that excess sulphur and amine are required in order to obtain a good yield (12). Therefore, the amount of quinaldine used in this reaction was increased to 1 mole. An oily product was obtained, from which only 0.5 % PTQA could be isolated. This low recovery was attributed to poor purification from the final reaction mixture. From the results of this investigation it was clear that the synthesis conditions and purification procedures had to be optimised.

3.2.2 Improving PTQA purification

The complex nature of the reaction product when PTQA is synthesised can be seen in Figure 3. 15. In order to simplify the mixture, vacuum distillation was used to remove unreacted quinaldine and amine before crystallisation of the product.

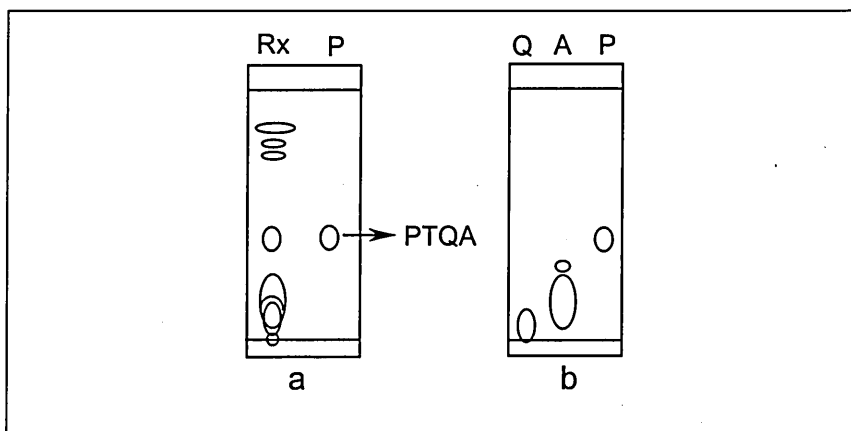


Figure 3. 15 TLC plates for the reaction mixture, a and reagents, b. Rx, P, Q and A denote reaction mixture, P PTQA, quinaldine, and 2- amino-pyridine, respectively.

Yields of about 1 % were obtained after a single vacuum distillation. Increasing the number of vacuum distillations to two did not lead to improvements in the yield. In order to improve the separation of the reactants from PTQA, column chromatography was used. Before the column separation was carried out, a suitable solvent had to be found, which was achieved by running the reaction mixture on TLC plates using solvents of different polarity. As shown in Figure 3. 16, chloroform and methylene chloride gave good separation, whereas with methanol, ethanol, isopropanol, hexane, and toluene no separation was achieved. Chloroform was therefore selected as the mobile phase for the column separation. The column showing the five coloured fractions is depicted in Figure 3. 17.

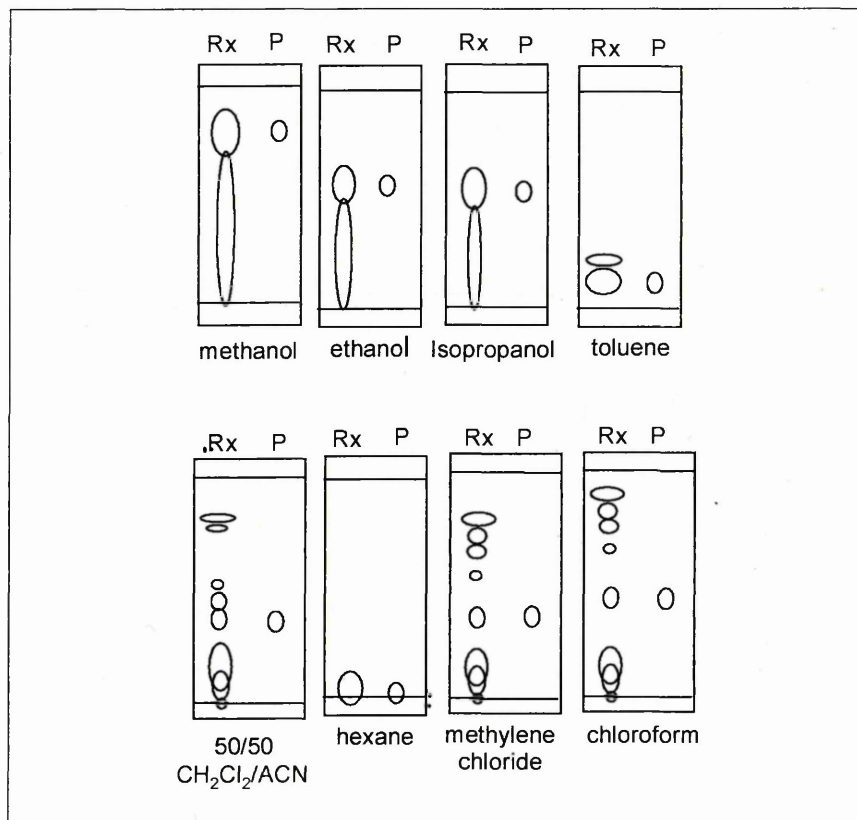


Figure 3. 16 TLC separations for the reaction mixture. P and Rx denote PTQA and the reaction mixture, respectively.

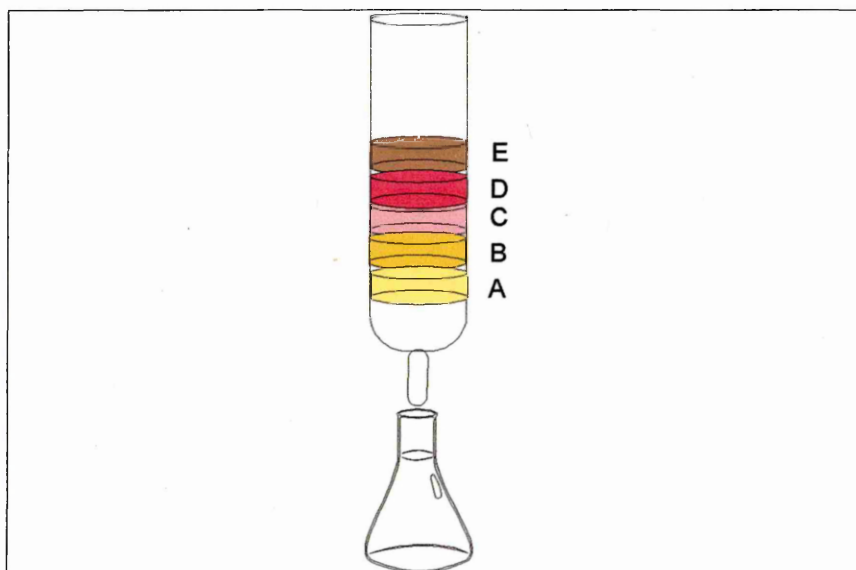


Figure 3. 17 Column separation of the reaction product separation was on a silica packed column with CHCl_3 as the mobile phase. The main fractions were: A elemental sulphur S_6 , S_7 , and S_8 , B PTQA and S_8 ; C PTQA, S_8 and one by-product, D trace of PTQA and by-products, E by products and reagents.

Identification of the main components in each fraction was by GC-MS.

Fraction A, was light yellow, eluted first from the column. Composed mainly of the cyclic sulphur allotropes, S₆, S₇ and S₈. The presence of these three compounds was confirmed from their corresponding spectra (see Figure 3. 18). In each spectrum the main mass correspond to S₂, which is at 64, while the compound mass 192, 224, and 256 were present in the S₆, S₇ and S₈ spectrum respectively.

Fraction B, was a dark yellow fraction and as shown in Figure 3. 19.a contains two compounds. The compound at 12.45 min has the same mass spectrum as S₈, while the main fragments (or ions) was identified as PTQA from the mass spectrum Figure 3. 19.b). The main components are at mass 78, 128, 137, and 265, which correspond to ⁺C₅H₄N, ⁺C₉H₆N, ⁺C₆H₅N₂S, C₁₅H₁₁N₃S, respectively.




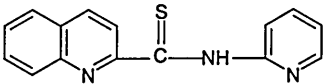
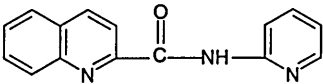
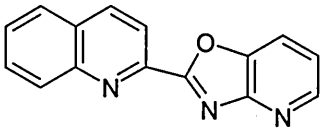
Fraction C, was a light pink-orange, and was found to contain three compounds as shown Figure 3. 20.a. The peak at 12.3 min correspond to S₈, the next peak at 16. 4 min is N-(α-pyridyl)-2-quinaldinamide (PQA), whose mass spectrum (Figure 3. 20.b), shows peaks at mass 78, 121, 128, and 249 which correspond to ⁺C₅H₄N, ⁺C₆H₅N₂O, ⁺C₉H₆N, and C₁₅H₁₁N₃O respectively. The third peak at 18.3 min, the major compound in this fraction, was identified as PTQA.

Fraction D, was red and it contained more than six peaks as shown in Figure 3. 21.a. The two major peaks were by-products, PQA at 16.4 min and its cyclic product at 16.5 min. The spectrum (Figure 3. 21.b) of the cyclic compound shows peaks at 77, 117, 247 corresponding to ⁺C₅H₃N ⁺C₆H₃N₂O, and C₁₅H₉N₃O. The peak at 18.2 min was identified as PTQA.

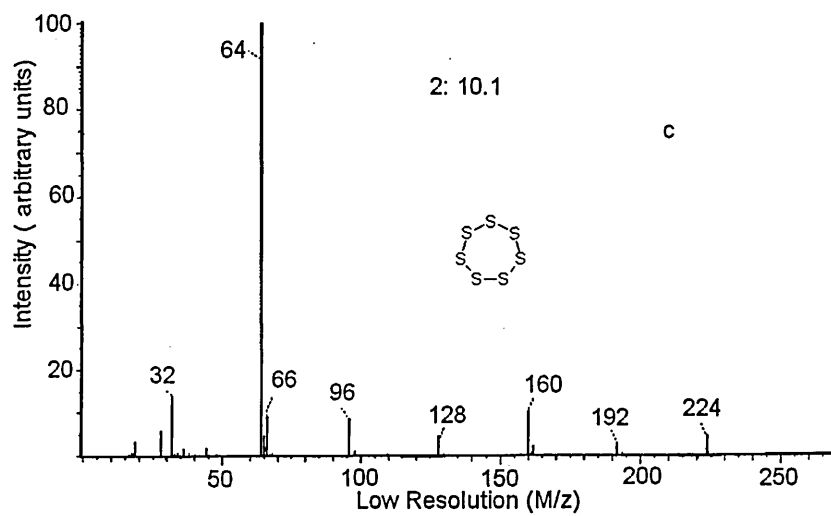
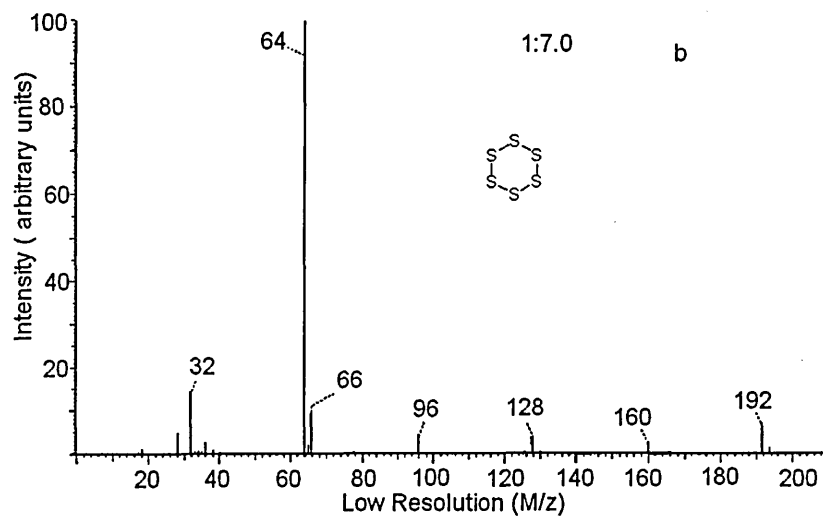
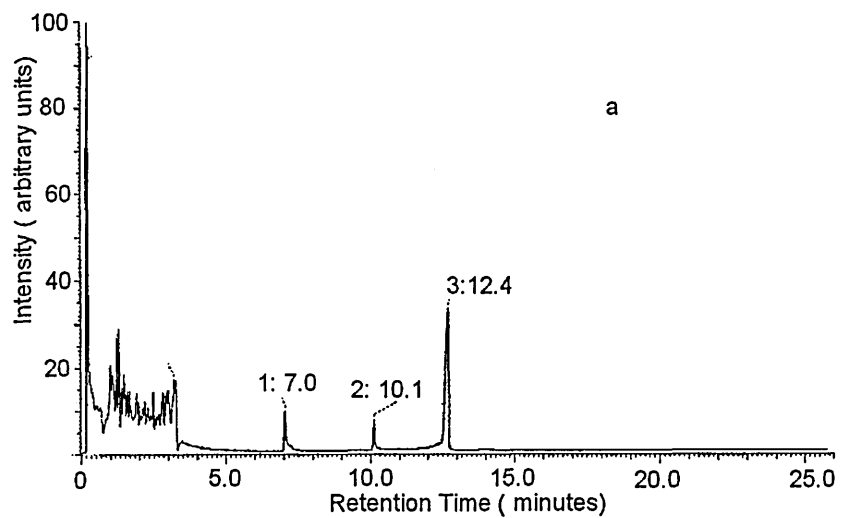
Fraction E, was a stronger red than the previous one; PTQA was not found in this fraction as shown in Figure 3. 22.a. The peak presented at 18.2 min presented a mass spectrum (Figure 3. 22.b), which did not correspond, to PTQA.

Table 3. 2 summarises the mass fragments used for the identification of each compounds.

Table 3. 2 Summary of the fragments used for the identification S₆, S₇, S₈, PTQA and two by-products.

Figure	Mass (m/z)	Main Fragments	Compound
3.18.b	64, 192	S ₂ and S ₆	
3.18.c	64, 224	S ₂ and S ₇	
3.19.b	64, 256	S ₂ and S ₈	
3.19.b	78, 128, 137, and 265	⁺ C ₅ H ₄ N, ⁺ C ₉ H ₆ N, ⁺ C ₆ H ₅ N ₂ S, and C ₁₅ H ₁₁ N ₃ S	
3.20.b	78, 121, 128, and 249	⁺ C ₅ H ₄ N, ⁺ C ₆ H ₅ N ₂ O, ⁺ C ₉ H ₆ N, and C ₁₅ H ₁₁ N ₃ O	
3.21.b	77, 117, 128, and 247	⁺ C ₅ H ₃ N, ⁺ C ₆ H ₃ N ₂ O, ⁺ C ₉ H ₆ N and C ₁₅ H ₉ N ₃ O	

In optimising the separation conditions a number of columns were examined. The column with the dimensions (12 cm x 60 cm) was found to give the best separation in which there was minimum overlap between the various fractions. Crystallisation of the pooled fractions B and C yielded yellow needles. Compared to vacuum distillation, the yield was 4.8 as against 1.3 %. The melting point of the product was 157 ± 2 °C, which is close to 155 ± 2 °C reported by Ahmed.



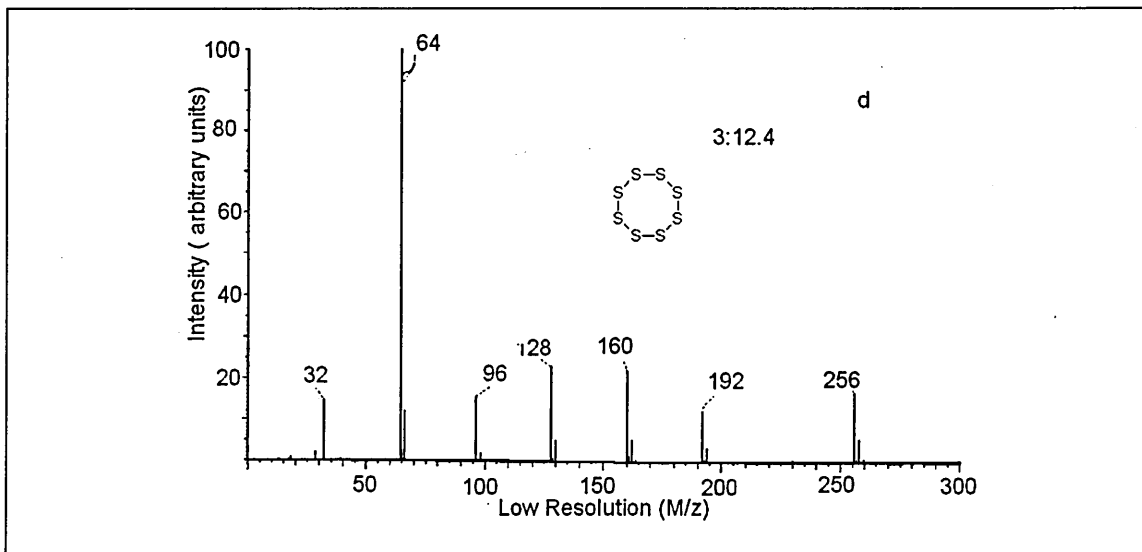


Figure 3. 18 Chromatogram (a) and mass spectra (b-d) of the constituents of fraction A eluted from the silica packed column. The mass spectra confirmed the presence of S₆, S₇ and S₈.

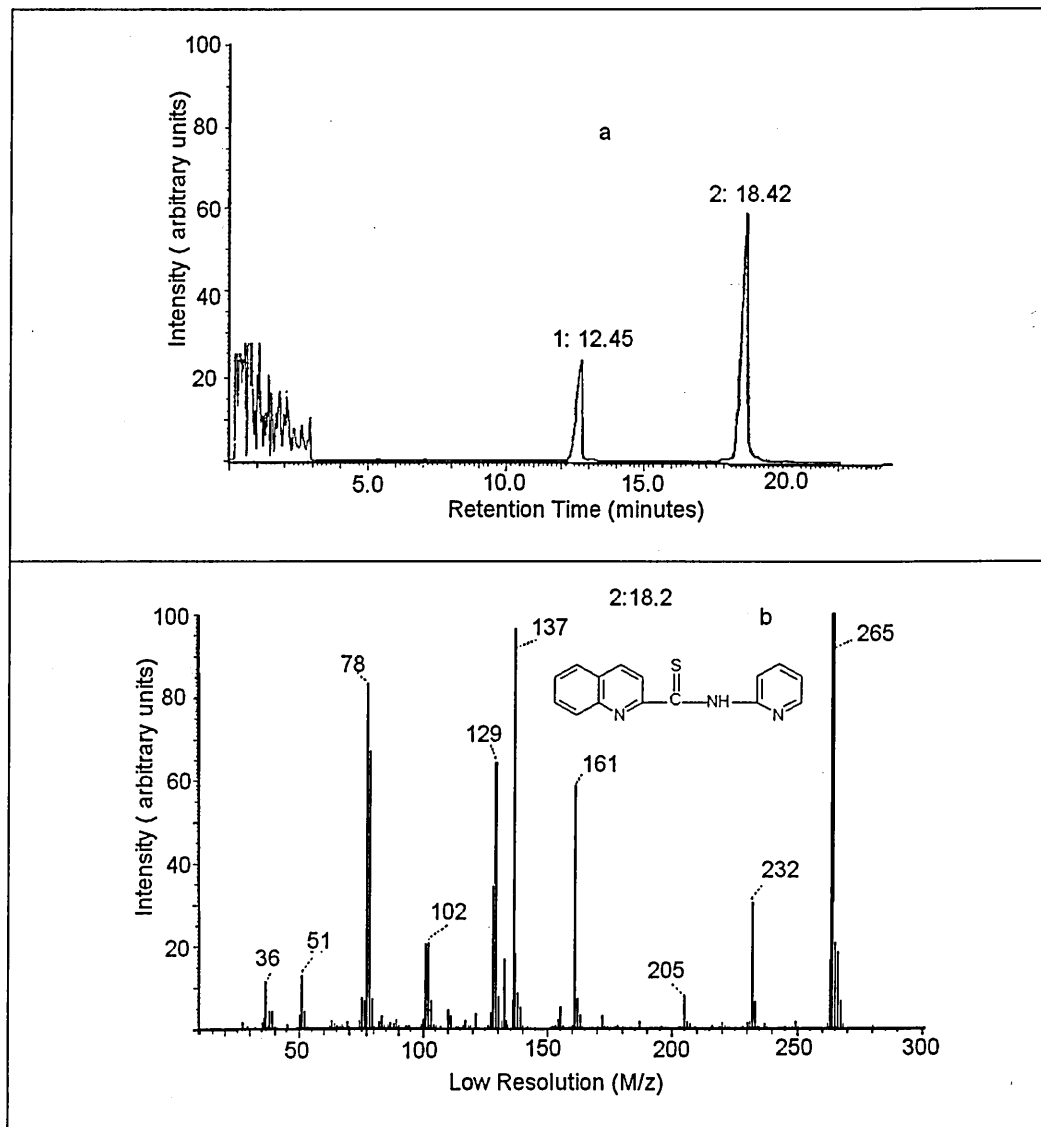


Figure 3. 19 Chromatogram (a) of fraction B eluted from the silica packed column and PTQA mass spectra (b).

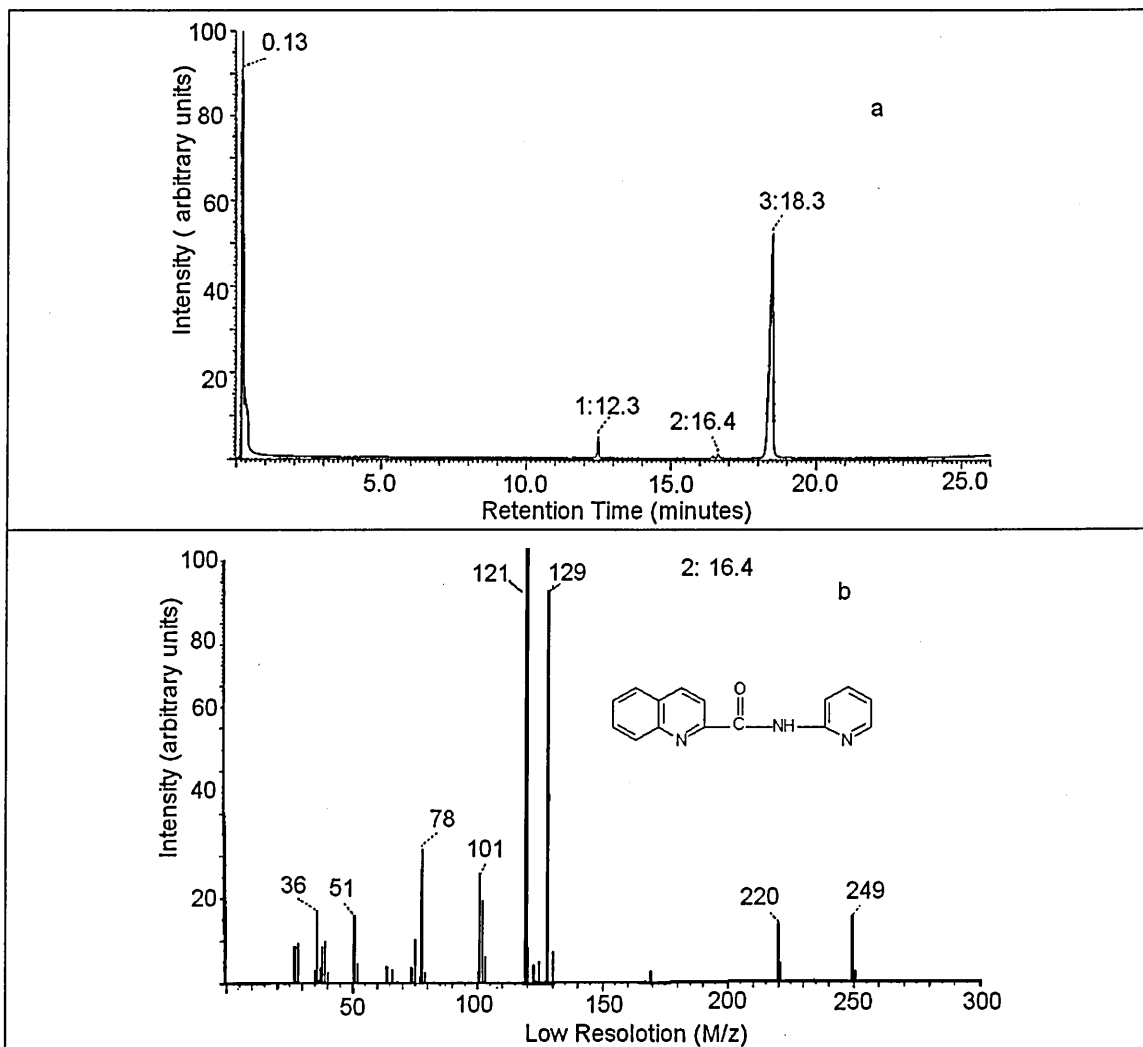


Figure 3. 20 Chromatogram (a) of fraction C eluted from the silica packed column and mass spectra (b) of the by-product with elution time of 16.40 minutes.

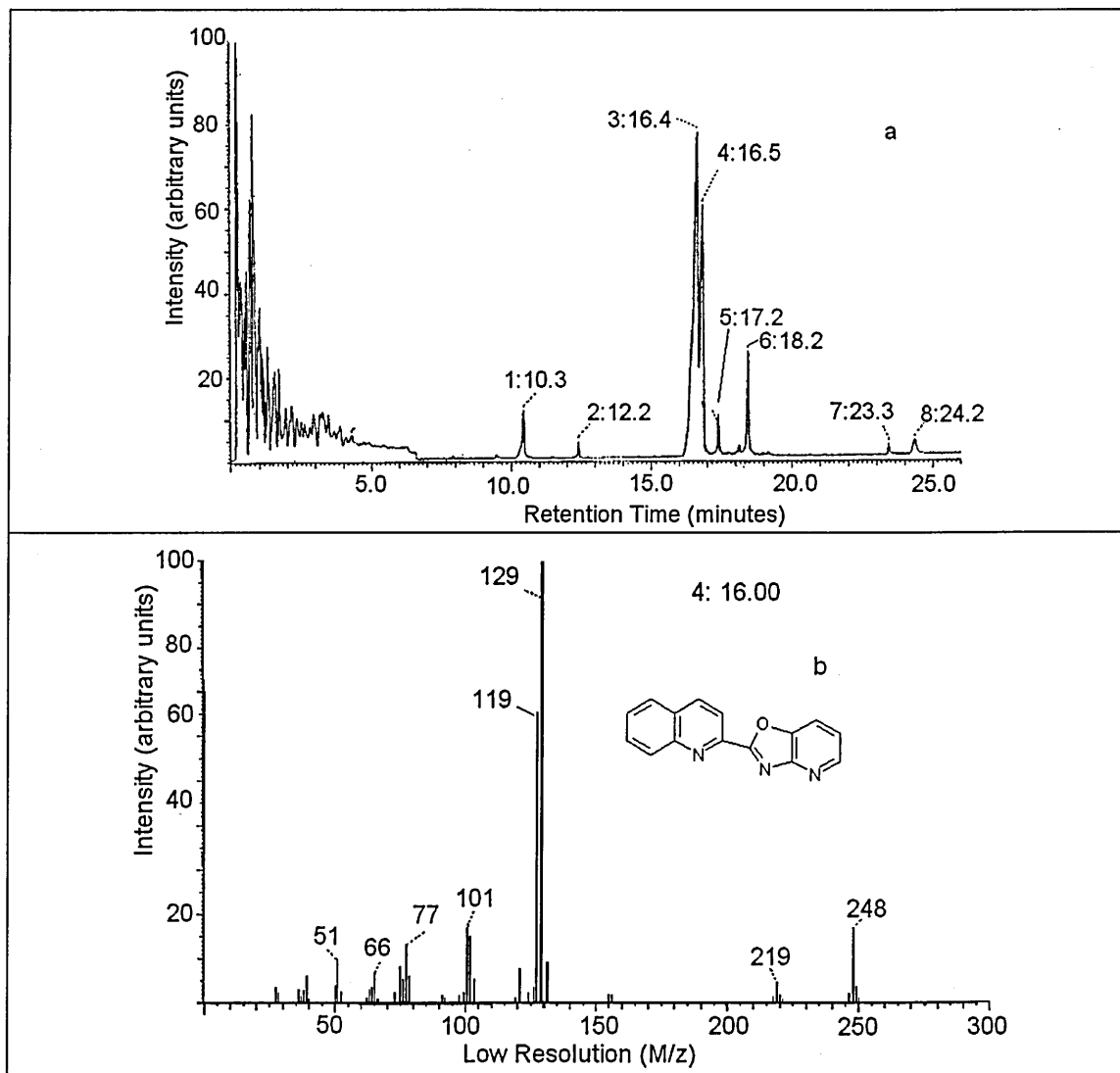


Figure 3. 21 Chromatogram (a) of fraction D eluted from the silica packed column and mass spectra (b) of the by-product with a retention time of 16.50 min.

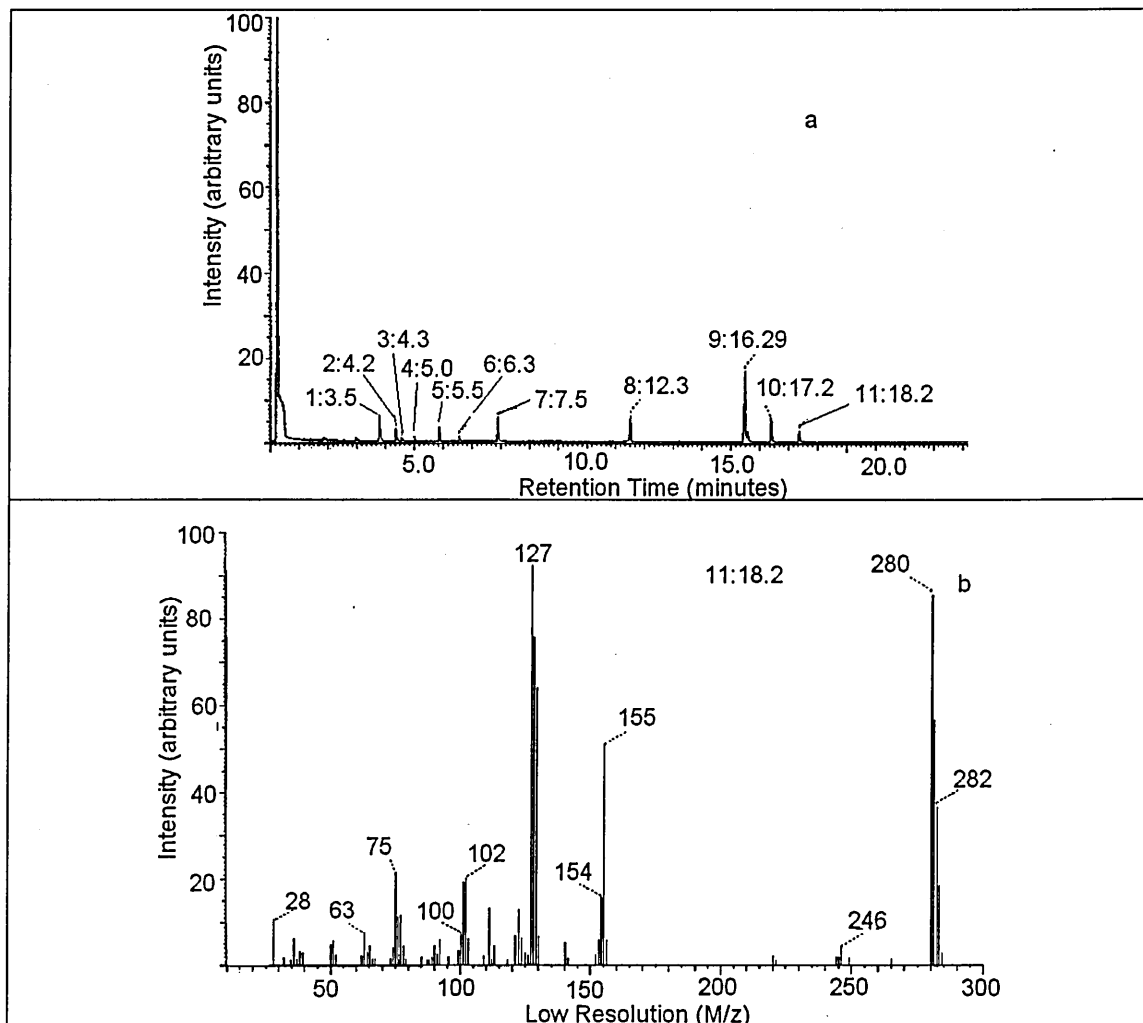


Figure 3. 22 Chromatogram (a) of fraction E eluted from the silica packed column and mass spectra of the compound with a retention time, 18.2 min similar to that the PTQA.

3.2.3 Use of Phosphorus pentasulphide (P_4S_{10}) for the synthesis of PTQA

For the synthesis of PTQA, quinaldine acid, phosphorus pentasulphide and 2-amino-pyridine in the presence of the solvent toluene were mixed and heated at 100 °C for 12 hr. Separation of the reaction mixture on a TLC plate showed the presence of one major component with an R_f of 0.33 (see **Figure 3. 24**).

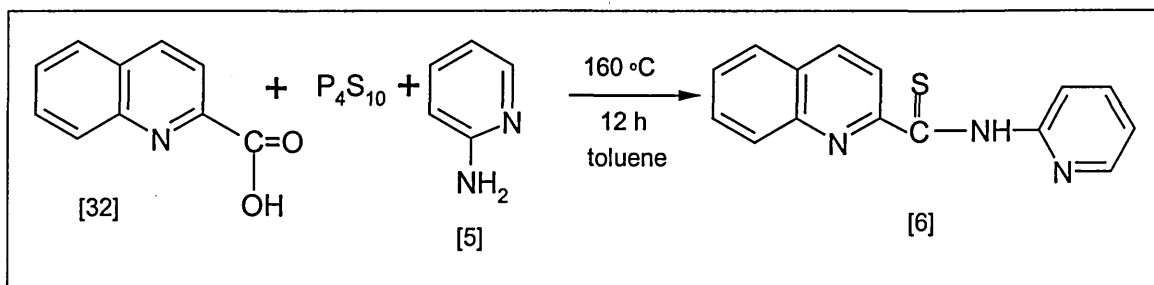


Figure 3. 23 PTQA Synthesis using quinaldine acid, phosphorus penta-sulphide, α -amino-pyridine in the presence of the solvent toluene at 100°C for 12 hr.

Isolation, and characterisation by GC-MS (Figure 3. 26) confirmed that this compound was the oxygen-containing analogue of PTQA [33], N- (α -pyridyl)-2-quinaldinamide (PQA). From this investigation it was found that PTQA could not be produced in one simple. To use this route, it is necessary to generate the carbonyl compound first and in a second step convert the carbonyl compound into a thio group.

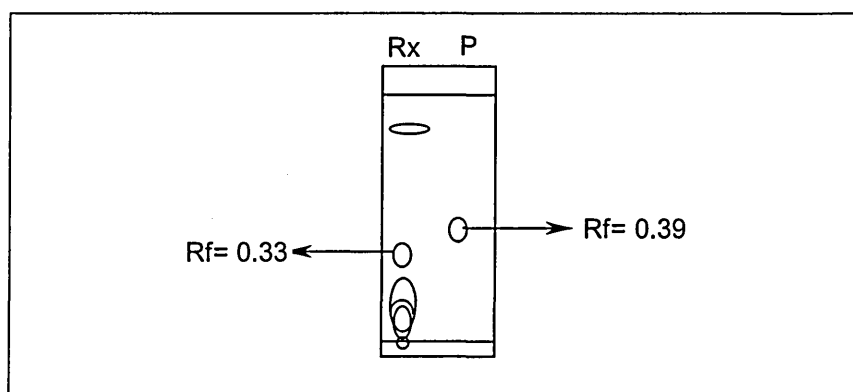


Figure 3. 24 TLC separation of the reaction mixture, Rx obtained for the synthesis of PTQA, and a sample of PTQA, P.

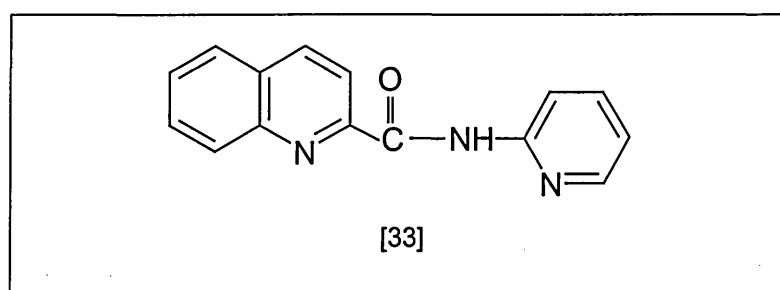


Figure 3. 25 The chemical structure of N-(α -pyridyl)-2-quinaldinamide PQA.

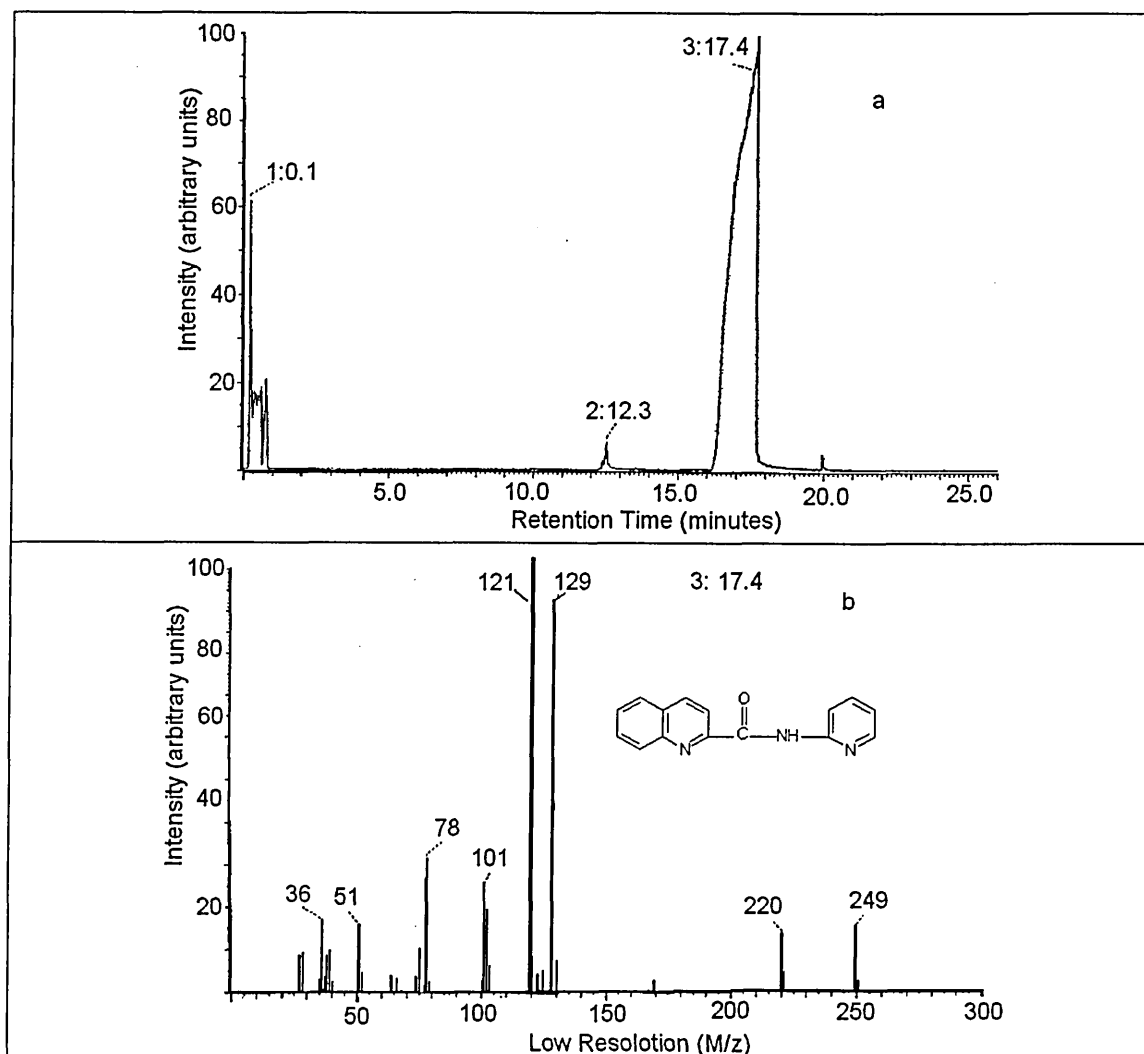


Figure 3.26 Gas chromatogram (a) and mass spectrum (b) of the product obtained when a mixture of quinaldine acid, phosphorus penta-sulphide, 2-amino-pyridine in the presence of the toluene heating at 160 °C for 12 hr.

3.2.4 Optimisation of the reaction time using the condensation reaction in the presence of sulphur

In order to optimise, the conditions for PTQA synthesis using the active methyl condensation reaction with 2-amine-pyridine in the presence of sulphur (**4**, **11**, **12**), reaction times were studied using the molar proportion 0.1: 0.20 :0.15 of quinaldine: 2-amino-pyridine and sulphur, respectively at 155 °C. Separation of the reaction mixture showed that PTQA had begun to form after 30 min. The reaction mixture was purified after 3, 6, 12 and 24 hours, and the yields are shown in Table 3.3. A slight decrease in yield from 8.5 to 7.8 % was observed when the reaction was allowed to continue for 24 hr instead of 12 hr.

Table 3. 3 The yield for the PTQA reaction using a molar ratio: 0.10:0.15:0.30 of quinaldine: 2-aminopyridine: sulphur, respectively, and heating the mixture at 155 °C for different set times.

Reaction Time (hours)	Reaction Yield (%)
3	2.8
6	4.7
12	8.5
24	7.8

3.2.5 The effect of the addition of Na₂S as a catalyst

The use of sodium sulphide as a catalyst for the condensation reaction as recommended in a Russian patent was investigated (13). 0.008 mole of Na₂S was added to a mixture of reagents containing 0.1, 0.2 and 0.3 moles of quinaldine, 2-aminopyridine and sulphur, respectively. The mixture was stirred and heated at either 120, 140, 150, or 160 °C for the set time of 3, 6, 12 or 24 hours. After the reaction time had elapsed, the mixture was set aside to cool. A glass column containing silica was used to separate the products. Yields from the column separation were 8 times higher than those obtained after vacuum distillation combined with crystallisation. Varying the heating temperature from 140 to 160 °C in steps of 5 °C showed that as the temperature increased to beyond 155 °C, the amount of PQA (fraction C) increased. Therefore the temperature was kept at 155 °C for the synthesis of PTQA.

Table 3. 4 The effect of the addition of sodium sulphide on the yield for the PTQA reaction using a molar ratio: 0.10:0.15:0.30:0.0080 quinaldine, 2-aminopyridine, sulphur, and sodium sulphide, respectively and heating the mixture at different set times and temperatures.

Temperature (°C)	Yield (%)			
	3 hr	6 hr	12 hr	24 hr
120	4.9	8.6	16.7	28.5
140	7.4	14.3	27.8	30.8
155	9.8	19.7	38.8	32.0
160	8.3	17.2	30.2	29.8

The addition of sodium sulphide resulted in much improved yields. After 12 hr, the yield was 38.8 as compared to 8.5 % when no sodium sulphide was added (see Table 3. 4). The yield of 38.8 % was lower than 80% reported in the patent. The difference in yield could be due to the size of the methyl compounds used in the synthesis. A smaller methyl-containing compound α -picoline was used in the patent.

3.1.4 Characterisation of PTQA

3.1.4.1 Elemental analysis (N, C, and H)

The elemental composition of PTQA was determined along with the data obtained from GC-MS measurements and melting point determination. The carbon, hydrogen and nitrogen contents were determined and compared with those obtained by Ahmed et al. (4) and the theoretical values (see Table 3. 5). The percentages found were lower than the theoretical values because of the presence of elemental sulphur.

Table 3. 5 Comparison of the carbon (C), hydrogen (H) and nitrogen (N) content in PTQA.

Element	Percent		Content (%)	
	Theoretical values	Reported by Ahmed (4)	Found	
N	15.8	13.4	15.8	
C	67.9	72.3	67.4	
S	12.1	NR	12.5	
H	4.2	4.3	4.0	

NR: no reported

3.3 CONCLUSION

The Ahmed synthesis of PTQA involved a 200 fold excess of sulphur and amine gave a yield of only 16 % after crystallisation. When the molar proportion used was 1.0, 1.5, 2.0 of quinaldine, sulphur and 2-aminopyridine, respectively, an oily complex mixture was obtained. After its purification by crystallisation, a yield of only 0.5% was obtained. The developed column separation improved the recovery of the product from the reaction mixture, as the yield of PTQA was increased from 0.5 to 5%.

For PTQA synthesis, the simplest and most efficient method is the methyl condensation of quinaldine with 2-aminopyridine in the presence of elemental sulphur using sodium sulphide to catalyse the reaction. The yield was increased 8 times, from 5 to 39 %, by the addition of Na_2S . The molar ratio used was 1.0, 1.5, 2.0, 0.08 of quinaldine, 2-amino-pyridine, sulphur, and sodium sulphide, respectively. However the oxygen-PTQA analogue is formed under these conditions. In order to resolve this problem, the reaction could be conducted under N_2 to avoid the presence of O_2 and in this way increase the yield of PTQA.

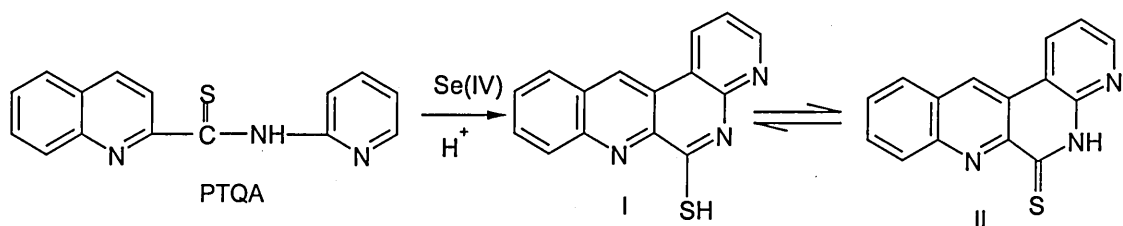
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Chapter 4:

Fluorescence Method for Total Selenium Determination



4.1 INTRODUCTION

Spectrofluorimetry is one of the commonly used techniques for the determination of trace selenium in a wide range of samples (1 - 8). Equipment for fluorimetric measurements is cheap and potentially the cost per sample preparation is low provided sample preparation is kept to a minimum. The literature on the determination of selenium using fluorimetry has been reviewed by Rodriguez et al (7). The fluorimetric reagent 2,3- diaminonaphthalene (DAN) is commonly used for selenium. Other compounds include Br₂DAN and PTQA. Hoste et al (9) introduced DAB, the first fluorimetric reagent used for the determination of selenium in 1948. Detailed investigation of DAB was carried out by Gillis in 1955 (9). DAB was used for four decades until it was superseded by DAN, which offers greater fluorescence sensitivity (8). However, the Se-DAN complex is not stable when exposed to light.

In 1988, Pal et al. (10) reported on 2- (α -Pyridyl) thioquinaldinamide (PTQA), a novel fluorimetric reagent that could be used for very sensitive fluorimetric determination of selenium (IV). The reaction of PTQA and selenium (IV) is based on the oxidation of the non-fluorescent PTQA in slightly acidic solution, 0.05-0.15 M sulphuric acid, to produce the fluorescent species, with excitation and emission wavelengths at 350 and 500 nm, respectively. PTQA produces the same spectral characteristics with excitation and emission wavelengths almost invariably around 350 nm and 500 nm, with chromium (VI), manganese (VII) and cerium (IV) and with persulphate, hydrogen peroxide, and triiodide in acidic media. This indicates that the fluorescent species is an oxidised product of the reagent itself and not a chelate. PTQA has many potential reaction sites and as a result the structure of the oxidised fluorescent species is difficult to predict. Given that ring closure can lead to intense fluorescence in some circumstances, it seems likely that photo-oxidative cyclisation takes place, leading to the formation of structure I in equilibrium with the tautomeric structure II as shown in the Figure 4. 1. The structure of the oxidised product has been confirmed by GC-MS (11).

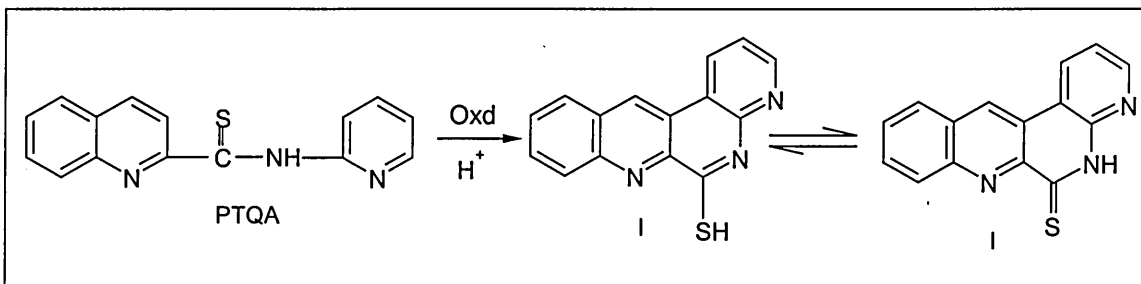


Figure 4. 1 Fluorescence process for PTQA in the presence of an oxidising agent in acidic medium.

As the PTQA and Se (IV) reaction is not specific since the cyclisation can occur in the presence of an oxidising agent (see Figure 4. 1) the effects of over sixty ions and complexing agents on the determination of 1 ng mL^{-1} selenium (IV) were studied individually by the authors (10). Table 4. 1 shows that there were no interferences on the selenium determination caused by species in categories 1 - 4. The interferences caused by species in categories 5 - 7 were eliminated using sodium tartrate or ammonium thiocyanate in the case of Cu (II), the interferences caused by Cu^{2+} , Ca^{2+} , Mg^{2+} could be due to an interaction between the ions and the PTQA structure.

Optimisation of the Se/PTQA ratio showed that the presence of excess reagent is not critical for the development of the fluorescence signal. Varying the Se/PTQA molar ratio from 1:20 to 1:200 in the presence of $10 \text{ } \mu\text{g L}^{-1}$ Se (IV) produced constant maximum fluorescence intensity. Five calibration curves covering the range 15 pg L^{-1} to $1 \text{ } \mu\text{g L}^{-1}$ were prepared. The developed method was applied to the determination of selenium in a sulphur sample, and synthetic mixtures.

Pal et al. (11) reported a direct spectrofluorimetric method for the determination of manganese in industrial and environmental samples, in which the reaction conditions, solvent, acid, time and the ratio of $[\text{PTQA}]/[\text{Mn (VII)}]$ were optimised. The main findings from the investigations showed that the fluorescence intensity peaked immediately after Mn (VII) was mixed with PTQA. Furthermore, the signal is stable for at least 72 hours, and the optimum acid medium was $0.83 \text{ M H}_3\text{PO}_4$. Linear response was obtained for the calibration graph covering a wide concentration range.

Table 4. 1 Possible interference effects on the PTQA-Se(IV) reaction caused by the presence of other ions.

Categories	Amount	Ions*	Comments
1	Several thousand-fold	tartrate or sulphate	No interferences
2	1000-fold	Alkali metals, Co (II), Ni (II), Se (VI), Au (III), NH_4^+ , citrate, oxalate, F^- , Cl^- , Br^- , I^- , NO_3^- , SCN^- , PO_4^{3-} , CH_3COO^- , persulfate, ClO_3^- , or azide	No interferences
3	100-fold	Tl (I), Rh (III), Hg (II), Ce (III, IV), EDTA	No interferences
4	50-fold	Cr (III, VI)	No interferences
5	500-fold, with Sodium tartrate	Al, Fe (II, III)	Interferences
6	50-fold, with Sodium tartrate	Ag (I), Be (II), Ca (II), Mg (II), Sr (II), Pb (II), Sn (II, IV), La, As (III), Th (V), Zr (V), Hf (V), Nb (V), Ta (V), Sb (V), V (V), Mo (VI), W (VI), U (VI)	Interferences
7	10-fold	Cu (II)	Interferences were masked with addition of ammonium thiocyanate

***Interference from permanganate or hydrogen peroxide was removed simply by adding sodium azide and boiling the solution.**

More recently, Ahmed et al (12) described a simple, sensitive and highly selective automated spectrofluorimetric method for the simultaneous determination of Se (IV) and Se (VI) by flow injection analysis (FIA). It was found that the fluorescent species was stable for 24 hours when it was prepared in 2.0 M H_2SO_4 containing ethanol and 1.0×10^{-4} M PTQA. Selenate reduction was carried out in an on-line photoreactor in the same acid medium. The linear dynamic range of the method was 0.01-2.2 $\mu\text{g mL}^{-1}$ Se (IV). The developed method was used for the determination of selenium in sediments, water, food (rice flour and egg) and human hair.

Subsequently, Pal et al. (13) developed selective and highly sensitive methods for the sequential determination of ultratrace amounts of Cr, Se and Mn in biological samples. Fluorescence species formed were stable for 24 hours. The linear dynamic

range of the method was $0.002 \mu\text{g mL}^{-1}$ to $0.9 \mu\text{g mL}^{-1}$. To avoid interference from Cr and Mn during the determination of the selenite species, Cr (VI) and Mn (VII) were reduced to Cr (III) and Mn (II), respectively.

A nonquenching fluorescent method based on the fluorescent species, Cr (VI)-PTQA, has been developed for the indirect determination of arsenic at nanotrace levels in alloys, underground water, industrial water and sewage sludge (14). A known excess of Cr (VI) was added to quantitatively oxidise As (III) in the presence of a mixture of iodide and chloride as catalyst followed by the addition of excess PTQA. Measurement of the fluorescence intensity were carried out at 488 nm and 360 nm, the emission and excitation wavelengths, respectively.

Paleologos et al. (15) developed a relatively simple, sensitive, and selective, automated fluorimetric method for the simultaneous determination of Cr (III) and Cr (VI) by flow injection analysis (FIA). The developed method was applied to environmental waters (mineral, tap and distilled), food samples (tomato juices) and reference materials.

More recently, Pal et al. (16) have shown that PTQA can be used for the fluorimetric determination of ruthenium at nanotrace levels. Fe (III) was added to catalyse the reaction carried out in 0.065 M H_2SO_4 , increasing the sensitivity. The range of linearity is $1\text{--}400 \mu\text{g L}^{-1}$ and a detection limit of $50 \mu\text{g L}^{-1}$. Interferences caused by the presence of other metals were removed with the addition of EDTA.

4.1.1 Sample pretreatment

In order to apply the fluorimetric method to solids, and solutions containing selenium in an oxidation state other than four, the samples have to be pre-treated. Rodriguez (7) has reviewed the wet digestion methods that can be applied to selenium-containing samples. Among the acid mixtures that have been used are $\text{H}_2\text{SO}_4/\text{HNO}_3$, $\text{HNO}_3/\text{HClO}_4$, $\text{H}_2\text{SO}_4/\text{HClO}_4$, and $\text{H}_2\text{SO}_4/\text{HNO}_3/\text{HClO}_4$. The use of perchloric acid is not recommended because of the risk of explosion. Successive digestions may be required in order to achieve protein digestion. Temperatures between 100 and 200°C are required for complete mineralisation. Microwave-assisted digestion is preferred, because the system is simple and easy to operate, and so can be adapted to a number of applications. It is essential during the sample pretreatment step that the element is not lost by volatilisation. As a result wet digestion is preferred. Wang (17) has described a simple microwave digestion

procedure for environmental and natural water samples using HNO_3 and a mixture of $\text{H}_2\text{O}_2/\text{H}_2\text{SO}_4$. A $\text{HNO}_3/\text{H}_2\text{O}_2$ mixture has been successfully applied to the microwave digestion of selenium supplements and food (7, 15).

The methods based on the fluorescence of the piaseenols (2,3-DAN or DAB) and Se-PTQA complex, require that the element is in the IV oxidation state, but after sample digestion the selenium may be in the form of selenate. In order to ensure that the lower oxidation state (IV) is formed, a reduction step must be incorporated into the sample pre-treatment step. One of the reduction steps commonly used method for Se (VI) is boiling the sample in either 6M or 4 M HCl. All the fluorimetric methods based on 2,3-DAN used HCl (4, 7). Recently, photoreduction has been used increasingly. Table 4.2 summarises the conditions used for photoreduction of selenium. The efficiency of the conversion of Se (VI) to Se (IV) increases with pH, as most of the authors report (18-21). Carvalho et al. (21) reported pH 10 as the optimum. However, Ahmed reported the photoreduction of Se (VI) in 2 M H_2SO_4 which a very acid medium. The mechanism for selenate photolysis is not clear as no studies of the process are available.

In this chapter, methods based on the fluorimetric determination of selenium using 2-(α -pyridyl)-thioquinadineamide (PTQA) and ICP-MS are compared after microwave-assisted digestion.

Table 4.2 Application of photoreduction to the determination of selenium, iron and chromium.

Reference	Element	Conditions
C. Measures and J. Burton (1980) (18)	Se	Medium pressure mercury lamp (1250 W), in 2.5 ml of 0.1 M Borax solution and approximately 50 μ L of 30 % H_2O_2 , the sample was irradiated for 5 hours at room temperature. Recoveries of about 86 % after sample reduction.
C. Van Den Berg and S. Khan (1990) (19)	Se	High pressure mercury lamp, UV-irradiation for 4 hour, the conversion was completed at pH 8.2 .
R. Liu (1992) (22)	Fe	High pressure mercury lamp, more than 95 % of Fe (III) can be reduced to (II) in the range of pH 3.0 – 7.0 (on-line system).
Batley (1996)(20)	Se	High pressure (550 W) and a low pressure (40 W) UV lamp were used in a basic medium, the photolysis shows that conversion is pH dependant. Under the same reduction conditions and the use of a low power lamp, 88 % reduction was achieved in 11 minutes at 30 °C. In contrast; high pressure lamp required 2 hour to complete the reduction.
M. Ahmed et al (1997) (12)	Se	High pressure mercury lamp, with 2 M H_2SO_4 solution at room temperature (on-line system).
Paleologos (1998) (15)	Cr	High pressure mercury lamp, with 2 M H_2SO_4 solution at room temperature (on-line system).
L. Carvalho and Col. (1999) (21)	Se	A commercial UV-Digester 705 (high pressure mercury lamp) was used in basic medium (adjusted with NaOH), the optimal pH was 10 with an irradiation time of 30 minutes at 90 °C.

4.2 RESULTS AND DISCUSSION

4.2.1 Optimisation of the conditions for the generation of the fluorescence signal

4.1.1.1 The choice of solvent

The PTQA reagent has low water solubility as a result it has to be dissolved in a non-aqueous solvent. In previous investigations with PTQA, ethanol has been the solvent of choice. In their studies, Ahmed et al (12) proposed the use of isopropanol for preparing the PTQA solution. In order to find the most suitable solvent, a 5×10^{-4} M PTQA solution was prepared in methanol (MeOH), ethanol (EtOH) and isopropanol (IsopOH), respectively. The fluorescence intensity for a $200 \mu\text{g L}^{-1}$ Se (IV) solution was found to increase with chain length from methanol to isopropanol, as shown in Figure 4.2. The signal increases, relative to methanol, were 14 % and 46 %, respectively. The higher signal in isopropanol could be because the PTQA is most soluble in this solvent. Besides obtaining the most intense signal in isopropanol, it is ten times less expensive than ethanol. As a result, isopropanol was used as a solvent for PTQA in all the subsequent experiments.

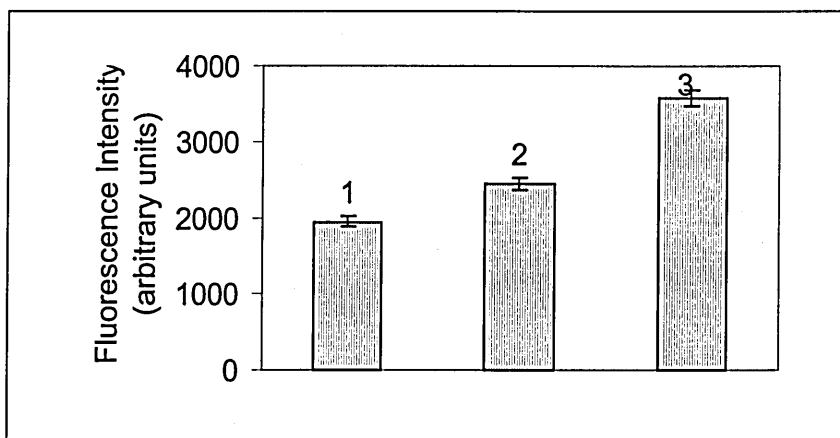


Figure 4.2 Comparison of the selenium signal intensities obtained when PTQA is dissolved in methanol (1), ethanol (2) and isopropanol (3), respectively.

4.2.1.2 Acid medium

In order to generate a fluorescence signal an acidic medium is required. The acid of choice in a number of investigations (15-21) has been sulphuric acid. Pal et al (16) compared the use of H_3PO_4 with H_2SO_4 , and in an earlier paper (10) by the same author the reaction was carried out in HCl. However, there have been no studies comparing the effects of different acids on the intensity of the selenium fluorescence signal. The following acids: HCl, CH_3COOH (HAc), H_3PO_4 , H_2SO_4 and HNO_3 were

investigated for their effect on the fluorescence signal. As shown in Figure 4. 4, the highest intensity signals were obtained when the reaction was carried out in HNO₃. The order of decreasing fluorescence intensity for the five acids was HNO₃ > H₂SO₄ > H₃PO₄ > HCl > HAc. This order can be explained in terms of the oxidation power of the individual acid. Since the reaction product formed is the result of photo-oxidative cyclisation, in acid medium, it is logical to expect that the use of a strong oxidation agent will lead to greater conversion and hence higher fluorescence intensity. Figure 4. 3 shows the oxidative cyclisation reaction catalysed by Se (IV), and responsible for the production of the fluorescence signal.

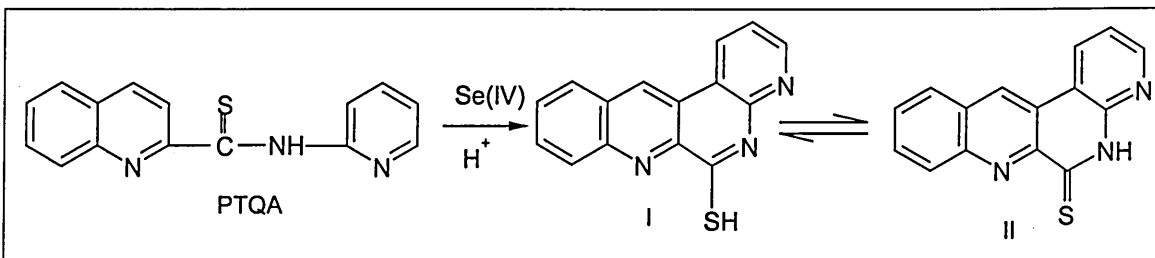


Figure 4. 3 The reaction of PTQA in the presence of Se (IV) in acidic medium.

There is not much to choose between H₃PO₄ and H₂SO₄, but the difference between H₂SO₄ and HNO₃ is remarkable. The difference in signal intensities when HNO₃ is used is about three times that in H₂SO₄.

4.2.1.3 Selection of the excitation wavelength

Measurements reported in the literature were carried out using either 350 nm or 360 nm as the excitation wavelength (λ_{exc}) and the fluorescence emission was detected at 500 nm. Figure 4. 4 shows that of the four excitation wavelengths investigated, 380nm gave the most intense signal for a solution containing 500 $\mu\text{g L}^{-1}$ selenium.

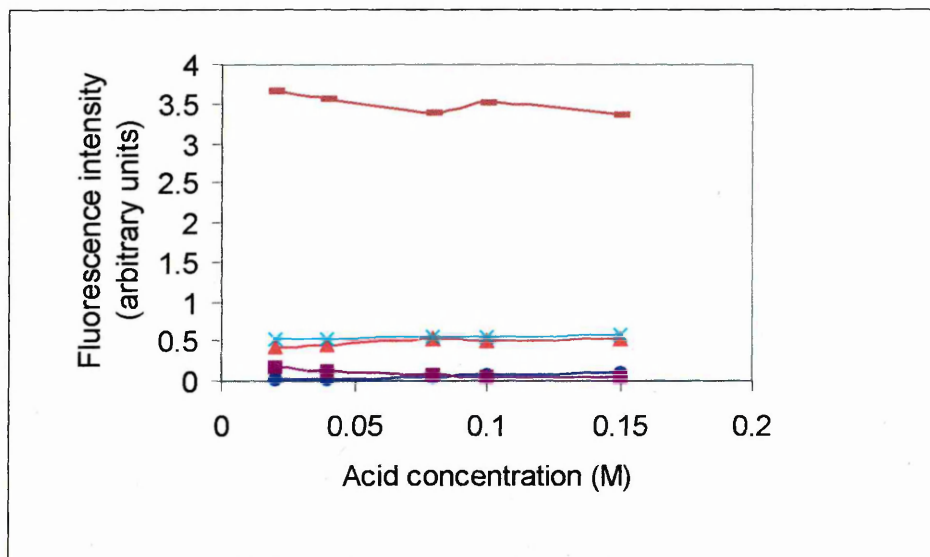


Figure 4. 4 Relative fluorescence intensities obtained for a $200 \mu\text{g L}^{-1}$ Se (IV) solution in 5×10^{-4} M PTQA in the presence of (—) HNO₃, (-x-) H₂SO₄, (-Δ-) H₃PO₄, (-■-) HCl and (-●-) HAc acids.

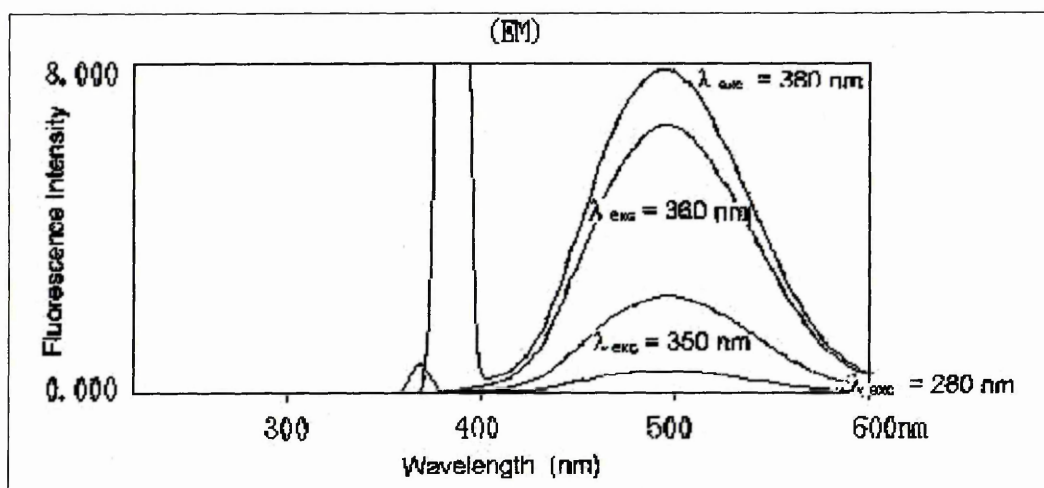


Figure 4. 5 Fluorescence signals obtained from $500 \mu\text{g L}^{-1}$ M PTQA and 0.1 M HNO₃ using 280, 350, 360 and 380 nm as excitation wavelength, respectively.

Calibration graphs obtained using 380nm as the excitation wavelength and varying the emission wavelength, showed that 500nm was the most sensitive detection wavelength, in agreement with the literature (Figure 4. 6).

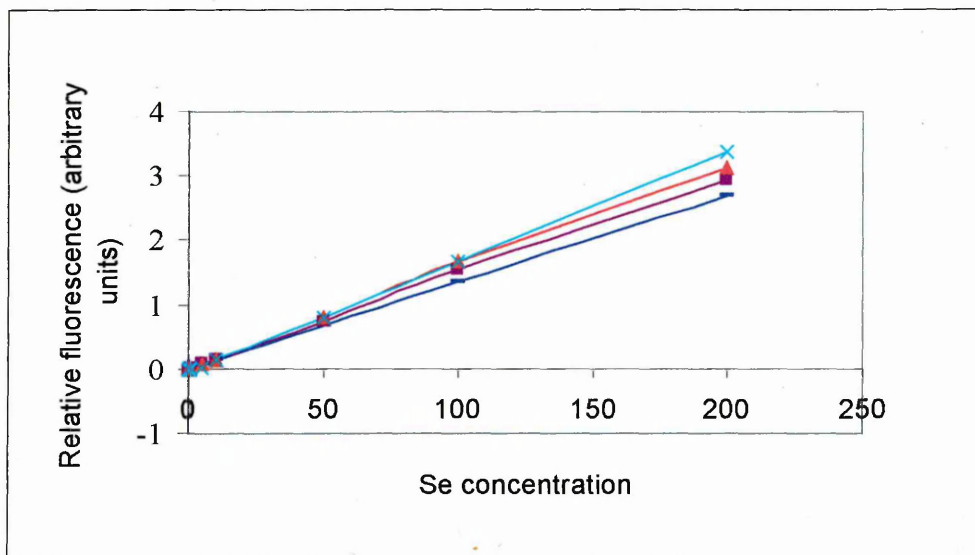


Figure 4. 6 Comparison of the slopes of the calibration graphs obtained at different emission wavelengths: 470 (—), 480 (- - -), 490 (-▲-) and 500 (-x-)nm, respectively.

4.2.2. Optimisation of the time for developing the PTQA-Se (IV) fluorescence signal

Results from previous studies suggest (10 - 15) that maximum fluorescence intensity is obtained immediately after mixing PTQA with selenium, and the signal intensity remains stable for more than 24 hours. The rate of the reaction was studied with a solution of $200 \mu\text{g L}^{-1}$ of Se (IV) in 0.1 M HNO_3 at two concentrations of PTQA, 5×10^{-4} M and 1×10^{-3} M, respectively. Fluorescence measurements were carried out at 5 minutes intervals until 90 minutes. As shown in Figure 4. 7, a stable signal was obtained after 25 minutes. Similar results were obtained when H_2SO_4 was used. The signal intensity was found to increase when the PTQA concentration was decreased from 1×10^{-3} to 5×10^{-4} M (Figure 4. 7). This result shows the close relationship between selenite and PTQA concentration. This close relationship could be due to the catalytic role of selenite in PTQA oxidation. Subsequent measurements were carried out using a 30 minutes waiting time and 5×10^{-4} M PTQA. The reagent solution was stable for a working day but when the solution was used after 24 hours the fluorescence intensity had decreased by more than 20 %. Therefore the PTQA solution was prepared daily.

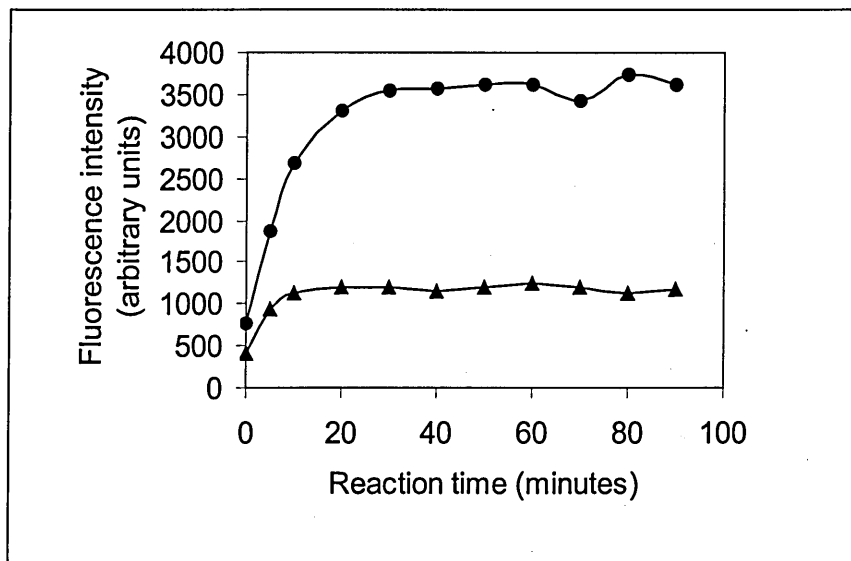


Figure 4. 7 Reaction time to optimisation using a solution of $200 \mu\text{g L}^{-1}$ Se (IV), 0.1M HNO_3 , $1 \times 10^{-3} \text{ M PTQA}$; a (- Δ -) and $5 \times 10^{-4} \text{ M PTQA}$; b (- \bullet -) in isopropanol.

4.2.3 Calibration curve

In order to determine the linear dynamic range of the developed method, solutions containing selenium concentrations ranging from 0.50 to $5000 \mu\text{g L}^{-1}$ were prepared in 0.1 M HNO_3 with $5 \times 10^{-4} \text{ M PTQA}$, and their fluorescence intensity measured after 30 minutes. Figure 4. 8 shows that there are three regions, a, b, and c that can be clearly identified, and a linear response from 0.50 to $500 \mu\text{g L}^{-1}$, was obtained in region, a. The second linear region, b is between 50 to $1000 \mu\text{g L}^{-1}$. In region c the instrument response does not change with concentration. The calibration graph obtained in region a is shown in Figure 4. 9.

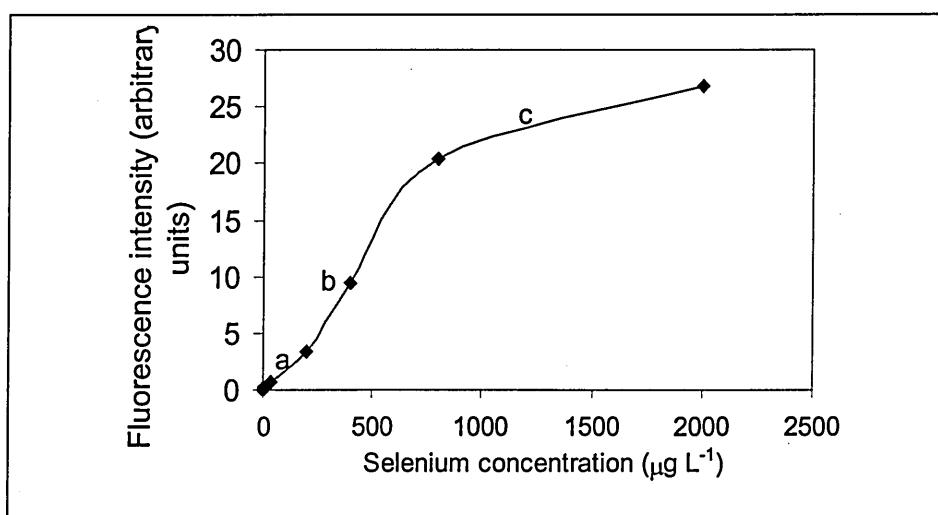


Figure 4. 8 The behaviour of selenium signal in the calibration range 0.4 to $5000 \mu\text{g L}^{-1}$ Se (IV).

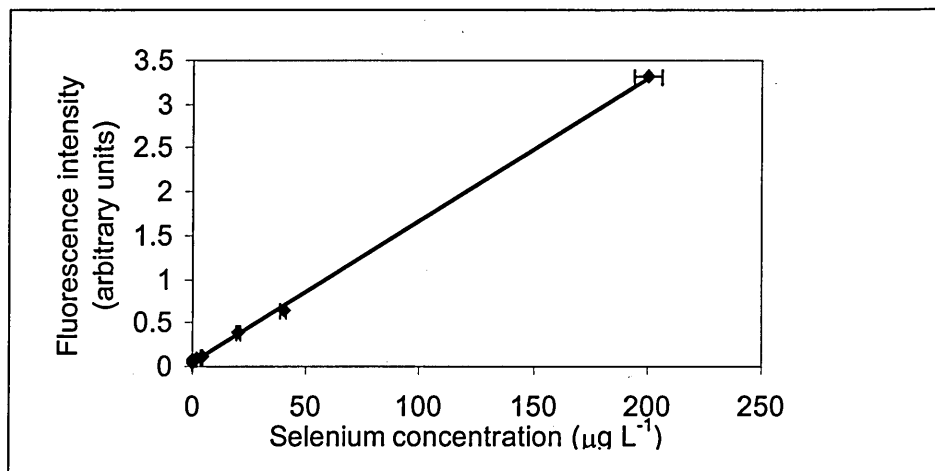


Figure 4. 9 Calibration curve for Se (IV) in the range 0.4 to 200 $\mu\text{g L}^{-1}$; $y = 0.0162x + 0.0521$ and $r^2 = 0.9996$.

4.2.4 Se (VI) reduction in the presence of HCl

Traditionally, HCl has been used to reduce selenate to selenite. The proposed reaction for the reduction is shown in Figure 4. 10.

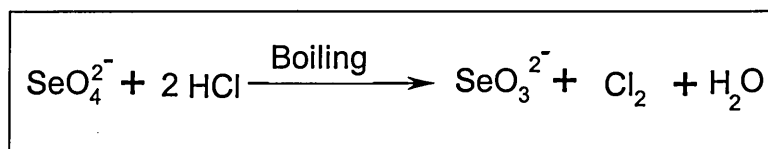


Figure 4. 10 Possible mechanism for the reduction of selenate to selenite in the presence of HCl.

Poor recoveries were obtained when solutions of 200 $\mu\text{g L}^{-1}$ Se (VI) was heated in 4 and 6 M, HCl respectively. Measurement of a solution containing Se (IV) showed a similar effect indicating that the presence of HCl may be causing signal depression, as shown in Figure 4. 11.

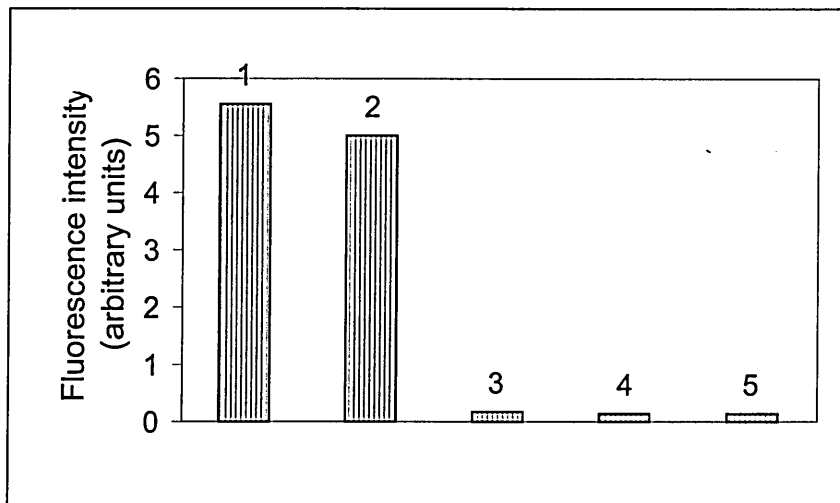


Figure 4. 11 Fluorescence signal intensity obtained in solutions containing: 1) Se (IV) in 0.1 M HNO₃, 2) Se (VI) +Se (IV) in 0.1 M HNO₃ and 0.01M HCl, 3) Se (IV) + Se(VI) in 0.1M HNO₃ and 0.5 M HCl, 4) Se (VI) in 0.1 M HNO₃ and 0.1 M HCl and 5) Se (VI) in 0.1 M HNO₃ and 0.5 M HCl. 500 µg L⁻¹ of each selenium species (Se (IV) and Se (VI)).

In contrast, Pal et al (10) found no interference when Cl⁻ is present at more than a 100 fold excess. However, it has been reported that fluorescence from quinolines and acridines, is quenched in the presence of halide ions such as Br⁻ and Cl⁻. Static quenching is characterised by complexation in the ground state between the interferent and the fluorescence molecule; as a result the complex does not fluorescence (23). The reduced signals for both Se (IV) and Se (IV) in the presence of HCl can be explained by the reaction of the halide with the quinoline group in PTQA. In order to demonstrate this quenching effect caused by the presence of halides, a solution of Se (IV) was prepared in different concentration of HCl, NaCl and NaBr. As shown in Figure 4. 12, the intensity of the fluorescence signal decreases with increasing halide concentration. The results of these experiments demonstrate that HCl could not be used as a reducing agent. Therefore the application of UV-photoreduction was considered.

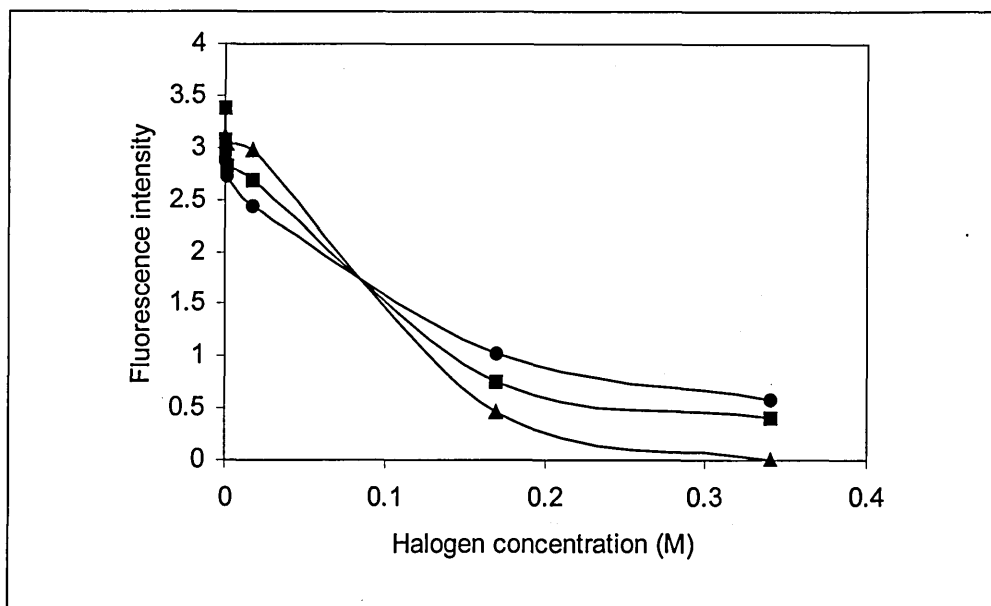


Figure 4. 12 Quenching effects of Cl^- and Br^- on the fluorescence signal from a $200 \mu\text{g L}^{-1}$ solution of Se (IV); (-●-) NaCl; (-■-) HCl; (-▲-) NaBr.

4.2.5 UV-Photoreduction

As mentioned earlier, selenate photoreduction is pH dependent with reaction commonly carried out at (18 -21), $\text{pH} > 7$. However Ahmed et al (12) reported the use of 2 M H_2SO_4 . Both acidic and basic conditions were investigated in order to establish the optimum photo-reduction conditions.

A comparison of the results obtained in acidic or basic media is summarised in Table 4. 3. The extent of conversion was greater in NaOH and also higher results were obtained with increasing NaOH concentrations.

Table 4. 3 The extent of conversion of Se (VI) to Se (IV) after photoreduction in increasing concentration of HNO_3 , H_2SO_4 and NaOH.

Concentration (M, mol L^{-1})	HNO_3	H_2SO_4	NaOH
0.1	< 10 %	< 10 %	18 %
1	< 10 %	< 10 %	28 %
2	< 10 %	< 10 %	32 %
2.5	-	-	32 %
5	-	-	38 %

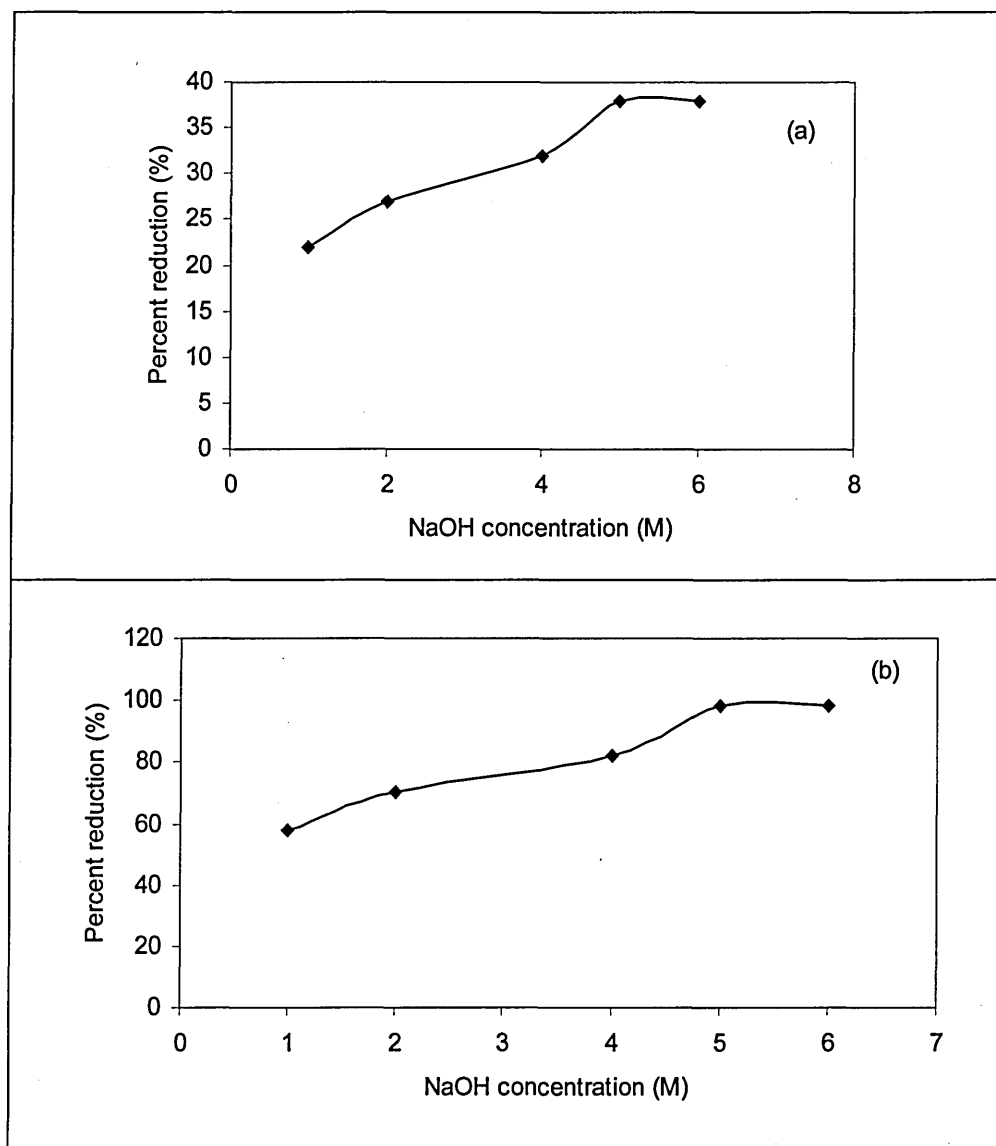


Figure 4. 13 Effect of NaOH concentration on the photoreduction of 200 µg L⁻¹ Se (VI) when the sample was irradiated 40 minutes at: (a) 25 °C and (b) 80 °C.

The increase in the extent of conversion in NaOH would indicate that the hydroxide ion may play a role in the photoreduction reaction. This observation is similar to that of Cavalho (21) who found that the rate of selenate photolysis increases with pH.

In a study by Daniels et al (24) and Shuali et al (25) of the photolysis of nitrate, they developed a reduction scheme similar to that proposed for selenate in Figure 4. 14

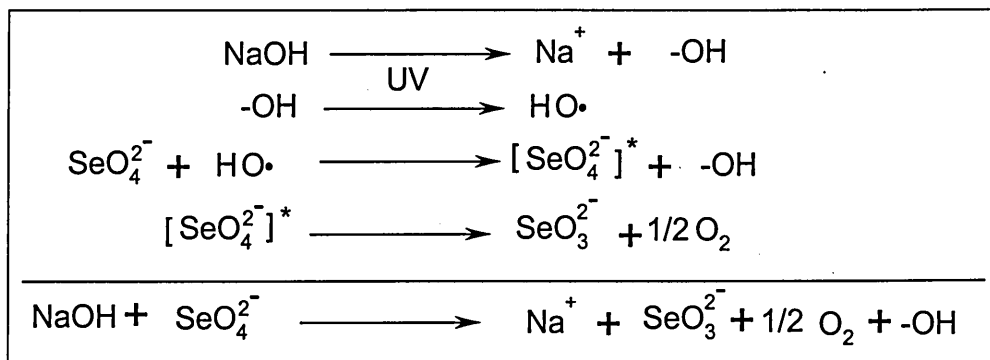


Figure 4. 14 Possible mechanism for the photoreduction of selenate in NaOH.

4.2.5.1 Effect of irradiation temperature on extent of photoreduction

As shown in Figure 4. 15, selenate conversion into selenite increased slightly with temperature. At 80 °C most of the Se (VI) was reduced to Se (IV). Consequently, 80 °C was used in subsequent measurements.

4.2.5.2 Effect of irradiation time on photoreduction

It was essential to establish the time required for photoreduction to reach completion. As shown in Figure 4. 16, when the reaction was stopped at five minutes a conversion lower than 20 % was obtained but with longer exposure times the amount of Se (VI) reduced to Se (IV) increased. As shown in Figure 4. 16, 98 % conversion was obtained after 40 minutes. No further improvements were obtained when the samples were irradiated for times longer than 40 minutes. It is important to note that selenite is oxidised to selenate at pH values greater than 7. Therefore it is essential to adjust the sample immediately after photoreduction to about 7. Under these conditions, the selenite species is stable for up to a day.

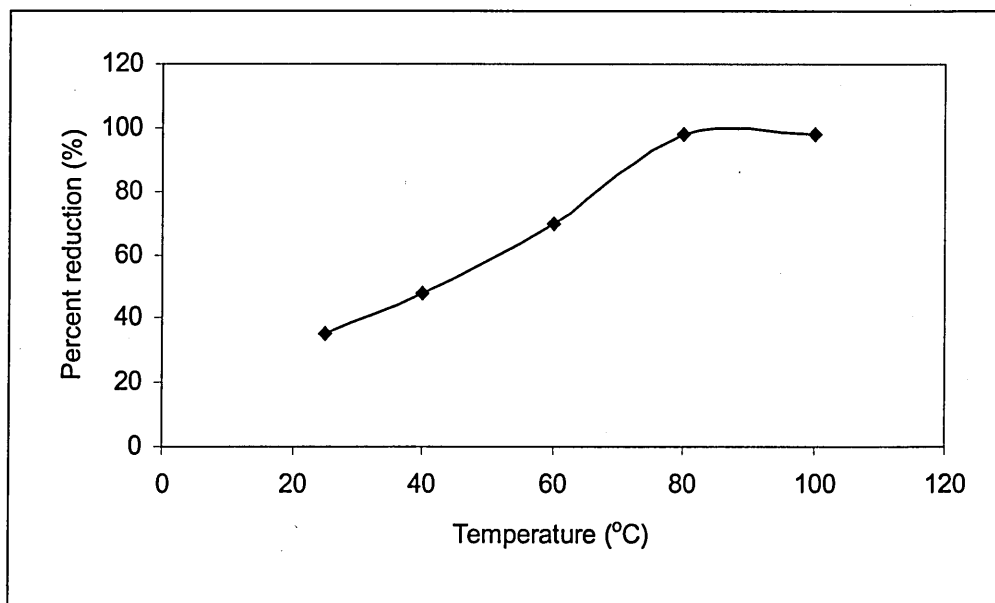


Figure 4. 15 Effect of temperature on the photoreduction of 200 µg L⁻¹ Se (VI) in 5 M NaOH after 40 minutes irradiation time.

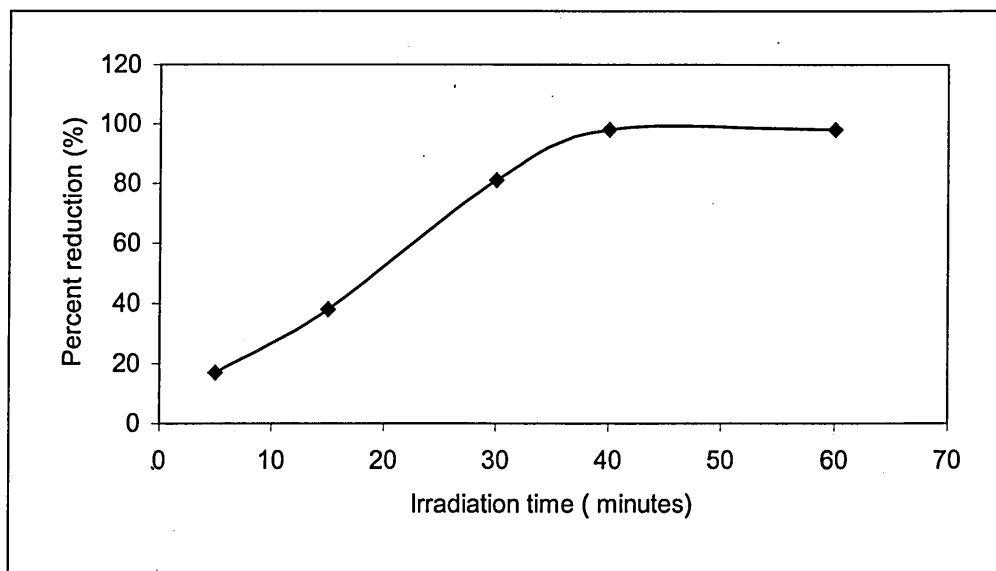


Figure 4. 16 Effect of the irradiation time on the photoreduction of 200 µg L⁻¹ Se (VI) to Se (IV) in 5 M NaOH at 80°C.

4.2.5.3 UV-Reactor Design

Once it had been established that photoreduction can be successfully applied, a home-made UV- photoreduction system was developed for the simultaneous treatment of five samples. The system consisted of five quartz tubes placed at equal distances from the low pressure mercury lamp (see figure 2.2, section 2.1.5, chapter 2). During digestion, the tubes were sealed with spring loaded glass stoppers. The stoppers were designed such that they can release excess pressure

generated in the tubes during the reaction. The whole system was kept at 80 °C in a water bath, made out of a steel beaker in order to prevent damage from the UV radiation. The total cost of the developed reactor was £ 1200 compared with £ 6000 for a commercial version. In addition, the system could easily be used in an on-line configuration.

Table 4. 4 summarises the optimum conditions for the determination of selenium by fluorescence.

Table 4. 4 Optimum parameters for the determination of selenium by molecular fluorescence.

Parameter	Optimum Condition
Fluorescence Method	
PTQA solvent	Isopropanol
PTQA concentration	5×10^{-4} M
Acid medium	0.1 M HNO ₃
Wavelength	$\lambda_{exc} = 380$ nm $\lambda_{ems} = 500$ nm
Time for maximum fluorescence intensity	30 minutes
Photoreduction	
Medium	5 M NaOH
Reduction time	40 minutes
Temperature	80 °C

4.2.6 Development of an off-line method for the determination of Se in nutritional supplements

Results of preliminary investigations showed that low selenium recoveries were obtained when the sample tablets are not pulverised before digestion. Furthermore, better recoveries were obtained when mixtures of H₂O₂ with either H₂SO₄ or HNO₃ are used for digestion.

A comparison of the effectiveness of H₂O₂, H₂O₂/H₂SO₄ and H₂O₂/HNO₃ to get selenium into solution is shown in Table 4. 5. Poorer recoveries were obtained for samples C and D in the H₂O₂/H₂SO₄ digestion solution.

Table 4. 5 Recoveries of selenium from nutritional supplements A, B, C, and D, selenomethionine, and selenocystine after digestion in either H_2O_2 , $\text{H}_2\text{O}_2/\text{H}_2\text{SO}_4$ or $\text{H}_2\text{O}_2/\text{HNO}_3$.

Sample	% Recovery		
	H_2O_2	$\text{H}_2\text{O}_2/\text{H}_2\text{SO}_4$	$\text{H}_2\text{O}_2/\text{HNO}_3$
A	83.8 ± 2.3	98.8 ± 1.9	101.2 ± 2.1
C	30.8 ± 1.4	63.6 ± 1.5	101.3 ± 2.7
D	45.6 ± 1.9	50.8 ± 1.1	101.6 ± 2.0
E	70.8 ± 1.7	96.8 ± 2.3	103.6 ± 1.8
Selenomethionine	89.7 ± 2.7	93.9 ± 1.8	98.6 ± 1.5
Selenocystine	89.1 ± 2.1	92.5 ± 1.6	98.0 ± 1.6

Once the photoreduction conditions have been optimised, it was clear that the acid digested samples could not be used directly. Sample pH must first be adjusted to 12 before photoreduction and subsequently neutralised to pH 7 to prevent the conversion of selenite to selenate. A schematic diagram of the steps in sample pretreatment is shown in Figure 4. 17. A comparison of the results obtained by the developed method and the expected selenium content in the nutritional supplements and samples of selenomethionine and selenocystine are presented in Table 4. 6.

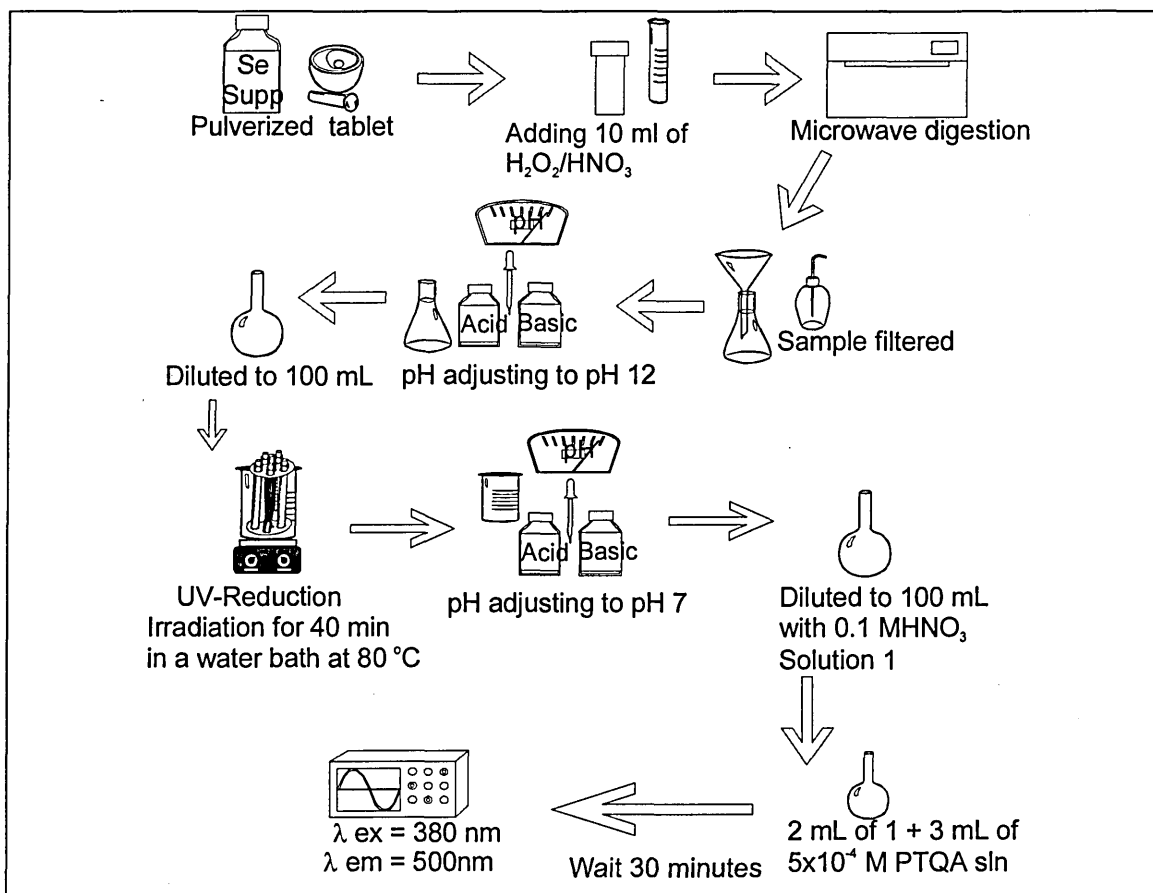


Figure 4. 17 Schematic diagram of the of the sample preparation steps used for the determination of total selenium in nutritional supplements.

Table 4. 6 Comparison of the expected results with those obtained using the developed fluorescence method.

Sample	Expected $\mu\text{g Se}$	*Fluorescence method $\mu\text{g Se}$
A	200.0	200.8 ± 1.6
B	25.0	25.3 ± 1.1
C	25.0	24.7 ± 1.1
D	100.0	100.9 ± 1.3
E	50.0	50.5 ± 1.6
F	25.0	25.6 ± 0.9
selenomethionine	884.4 ± 40.2	866.2 ± 22.9
selenocystine	1000.4 ± 47.2	982.9 ± 19.4
Reference	12.0 ± 3.0	12.3 ± 0.4
51.2		

for A, D, E and Reference solution (n= 10) and for B, C, F, selenomethionine and selenocystine (n=5).

4.2.7 Validation of the fluorescence method using a developed ICP-MS method

In order to validate the fluorescence method, a procedure based on ICP-MS detection was developed. The microwave digested samples were analysed directly instead of after the photoreduction step. The reason for this was to prevent both physical and chemical interferences caused by the presence of high sodium content from the added sodium hydroxide used to adjust the sample pH. However, the use of non-matrix material standards resulted in higher values being obtained for the samples as shown in Table 4. 7. In order to improve both accuracy and precision, standards containing sodium and calcium concentrations as in the samples, and an internal standard were used. Table 4. 8 shows the results obtained with matrix-matched standards, and yttrium or indium used as an internal standard. The difference between the results when yttrium or indium was used as internal standard was not statistically significant at 95 % confidence limits. Indium was selected and used in all subsequent measurements.

Table 4. 7 Results of selenium determination by ICP-MS without the use of matrix matched standards.

Sample	Amount of Se expected (µg)	Amount of Se obtained (µg)
A	200.0	152.3 ± 4.2
D	100.0	266.0 ± 4.8
E	50.0	63.7 ± 6.0

Table 4. 8 The results of determination of selenium by ICP-MS using matrix-matched and material standards

Sample	Expected (µg) Se	Found (µg) (Yttrium)	Found (µg) (Indium)
A	200.0	210.6 ± 10.6	202.2 ± 11.4
C	25.0	24.6 ± 3.5	24.8 ± 4.3
D	100.0	108.7 ± 8.8	106.5 ± 8.7
E	50.0	47.6 ± 4.8	48.1 ± 3.4
Selenomethionine	1200.0	1217.7 ± 22.8	1220.0 ± 20.7
Selenocystine	1200.0	1209.8 ± 20.9	1218.1 ± 21.3

After microwave digestion an appropriate dilution of the sample was introduced into the ICP-MS and the calculated recoveries for each sample using either H₂O₂, H₂O₂/H₂SO₄ or H₂O₂/HNO₃ for the digestion are shown in Table 4. 9. The highest recoveries were obtained for selenium when H₂O₂/HNO₃ was used for the digestion of the samples analysed by the fluorimetric method.

Table 4. 9 Recoveries of selenium from nutritional supplements, selenomethionine and selenocystine after digestion with either H₂O₂, H₂O₂/H₂SO₄ or H₂O₂/HNO₃ selenium determination was by ICP-MS.

Sample	% Recovery		
	H ₂ O ₂	H ₂ O ₂ /H ₂ SO ₄	H ₂ O ₂ /HNO ₃
A	89.0 ± 1.8	95.0 ± 2.0	102.8 ± 3.2
C	14.9 ± 0.8	72.6 ± 0.7	103.6 ± 2.7
D	39.1 ± 1.5	58.5 ± 1.1	103.6 ± 2.9
E	74.7 ± 0.3	97.1 ± 1.2	100.1 ± 3.5
Selenomethionine	86.4 ± 1.3	97.1 ± 1.0	97.7 ± 1.8
Selenocystine	83.9 ± 1.8	95.0 ± 1.0	98.0 ± 1.5

Table 4. 10 Comparison of the expected results and the obtained results for the determination of selenium by ICP-MS.

Sample	µg Se expected	µg Se ICP-MS
A	200.0	199.6 ± 9.8
B	25.0	26.1 ± 1.3
C	25.0	24.7 ± 0.4
D	100.0	102.9 ± 4.7
E	50.0	51.4 ± 3.61
F	25.0	25.9 ± 1.8
Selenomethionine	884.4 ± 40.2	868.8 ± 18.3
Secystine	1000.4 ± 47.2	986.2 ± 18.2
reference solution	4.2 ± 1.3	4.14 ± 2.3

for A, D, E and Reference solution (n= 10) and for B, C, F, selenomethionine and selenocystine (n=5).

Table 4. 10 shows the results obtained by the developed ICP-MS method for the determination of the selenium in a number of nutritional supplements, selenomethionine and selenocystine. Comparison of the expected values with the results obtained show that the differences were not statistically significant at the 95 % confidence interval.

Table 4. 11 Comparison of the results obtained using the developed fluorescence method with ICP-MS.

Sample	Expected $\mu\text{g Se}$	$\mu\text{g Se ICP-MS}$	Fluorescence method $\mu\text{g Se}$
A	200.0	199.6 ± 9.8	200.8 ± 1.6
B	25.0	26.1 ± 1.3	25.3 ± 1.1
C	25.0	24.7 ± 0.4	24.7 ± 1.1
D	100.0	102.9 ± 4.7	100.9 ± 1.3
E	50.0	51.4 ± 3.61	50.5 ± 1.6
F	25.0	25.9 ± 1.8	25.6 ± 0.9
Selenomethionine	884.4 ± 40.2	868.8 ± 18.3	866.2 ± 22.9
Selenocystine	1000.4 ± 47.2	986.2 ± 18.2	982.9 ± 19.4

for A, D, E and Reference solution (n= 10) and for B, C, F, selenomethionine and selenocystine (n=5).

4.3 CONCLUSION

A higher intensity fluorescence signal from Se (IV)-PTQA was obtained with HNO_3 and isopropanol as the medium. From the range of solvents and acids studied, the order of fluorescence intensity was follows: Isopropanol > Ethanol > Methanol, and $\text{HNO}_3 > \text{H}_2\text{SO}_4 > \text{H}_3\text{PO}_4 > \text{HCl} > \text{HAc}$, respectively.

30 minutes was found to be the optimal time for the development of the fluorescence signal. A liner range from 0.4 to $1000 \mu\text{g L}^{-1}$ was found for Se (IV).

The use of HCl or HBr as a reducing agent for Se (VI) was found to interfere in the fluorescence determination due to the quenching of the fluorescence of PTQA by the ions Cl^- and Br^- . For the reduction of Se (VI) to Se (IV) the use UV irradiation in a basic medium was effective, the rate depending on pH. Using 5 M NaOH solution, with 40 minutes of irradiation at 80°C , gave a 98% reduction of selenate. After irradiation, the pH should be reduced to 7 in order to avoid the oxidation of Se (IV). The developed system for the simultaneous irradiation of selenate samples was shown to be effective and to save analysis time.

The microwave digestion using the mixture $\text{H}_2\text{O}_2:\text{HNO}_3$ give a selenium recovery of 99% for each sample.

The analysis of selenium supplements by the developed methods shows an excellent agreement between the results from by the fluorescence method and those from ICP-MS method.

4.4 REFERENCES

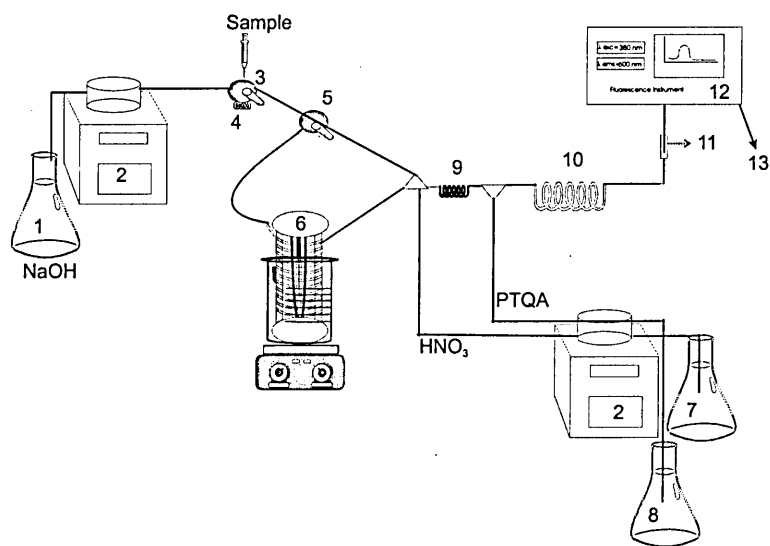
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Chapter 5:

On-line System for the Determination of Se (IV) and Se (VI)



5.1 INTRODUCTION

For the routine determination of selenium, a method that is simple and with a high sample throughput is required. One of the techniques that has been used for automated analysis is flow injection analysis (FIA) developed by Ruzicka and Hansen (1) in 1978. FIA is based on the injection of liquid sample into a moving, non-segmented continuous carrier stream, which transports the analytes to the detector. On-line sample preparation can be carried out on a FIA system. A key component of such a system is the detector. Over the last 20 years, a number of techniques have been used as on-line detectors for the determination of selenium. Figure 5. 1 gives a breakdown of the percentage use of each type of technique that has been used for selenium detection.

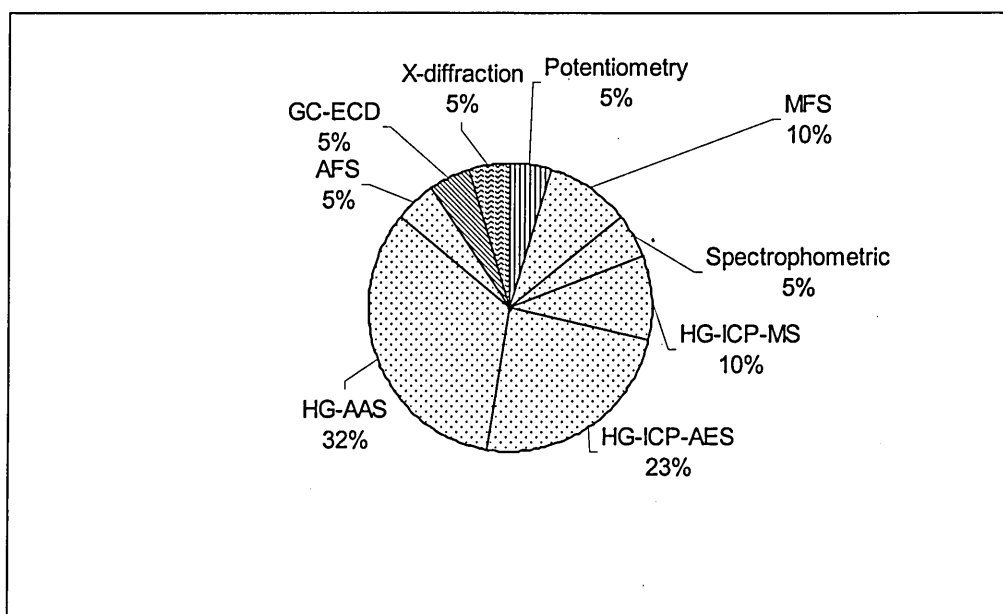


Figure 5. 1 Detection techniques used for on-line methods developed in the last 20 years for selenium determination (2-13).

As can be seen atomic absorption spectroscopy (AAS) with or without hydride generation (HG) is the commonly used technique (2 -5). Other techniques include ICP-AES (6 -9), ICP-MS (9, 10) and atomic fluorescence (AFS) (11). Molecular spectrometry accounts for only 15 % of usage, of which molecular fluorescence (MFS) contributes 10% (12, 13). One of the first automated methods for the determination of selenite and selenate after separation on a HPLC column was reported by Shibata et al (12). On-line selenate to selenite reduction was achieved with the use of HBr at 100 °C. Using the set-up shown in Figure 5. 2, the authors were able to form the Se (IV)–DAN complex and extract it into an organic phase before detection.

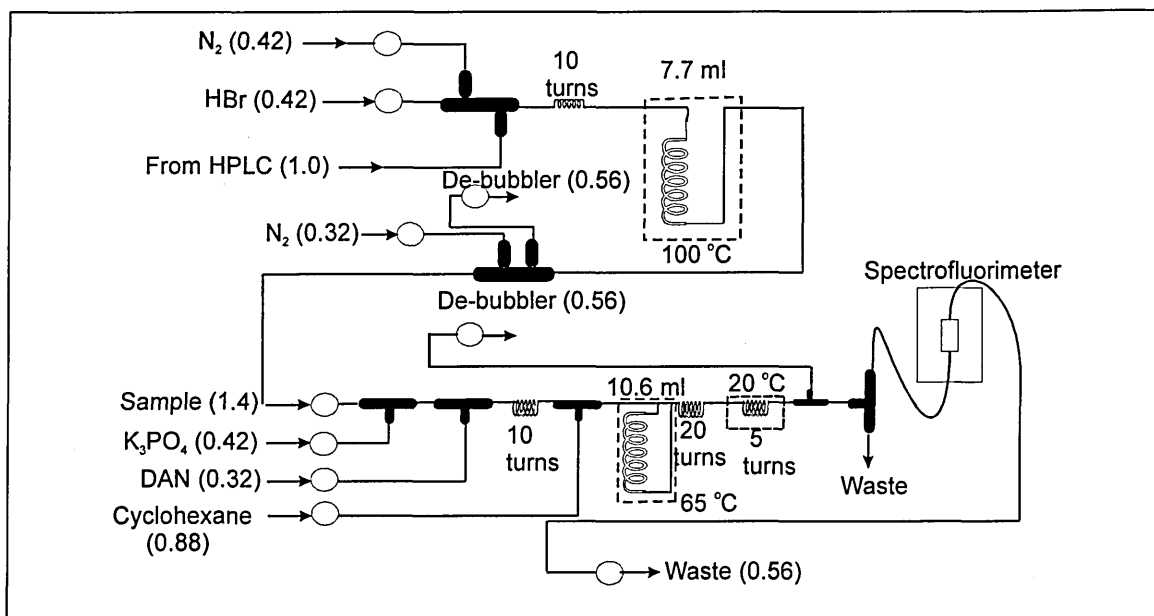


Figure 5. 2 From Shibata and co-worker (12). Flow diagram of the autoanalyser-based selenium detection system. The numbers in parentheses are the flow-rates for each line (ml min^{-1}). The concentrations of the stock solutions are: HBr, 6N; K_3PO_4 , 2M; DAN, 0.2% m/V in 0.05 N HCl.

This system was based on one developed earlier by Brown et al (14), for the determination of selenite in which no reduction step was included in the manifold (Figure 5. 3). The water bath was covered so as to prevent dissociation of the Se (IV)-DAN complex. Compared to the manual method this was found to be a big improvement, being more rapid and less laborious.

More recently, Ahmed et al (13), developed an on-line method based on the formation of Se (IV)-PTQA. The system used is shown in Figure 5. 4. The sample containing Se (IV) is injected into a 2 M H_2SO_4 stream, which is mixed with an alcoholic solution of PTQA. Solutions containing Se (VI) were first reduced in the photoreduction unit before PTQA was added, followed by fluorescence detection. Using this system, it was possible to determine total selenium and selenite levels, and the selenate concentration is found by difference.

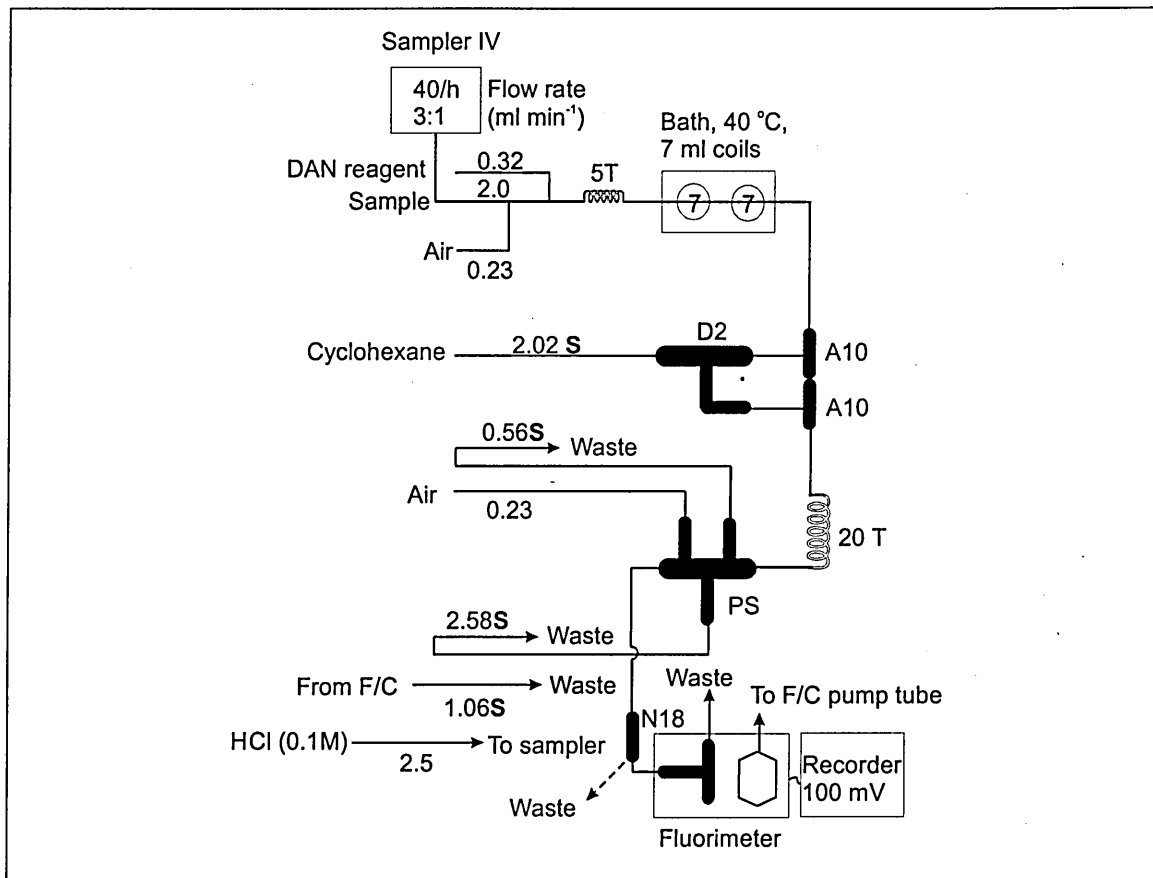


Figure 5. 3 From Brown et al. auto-analyser manifold. S after flow rate indicates solvents solvaflex tubing. F/C denotes flow cell. PS denotes a 5-point phase separator with Teflon insert. Transmission line from separator to fluorimeter is polythene (i.d. 0.03 in). All other transmission lines are glass.

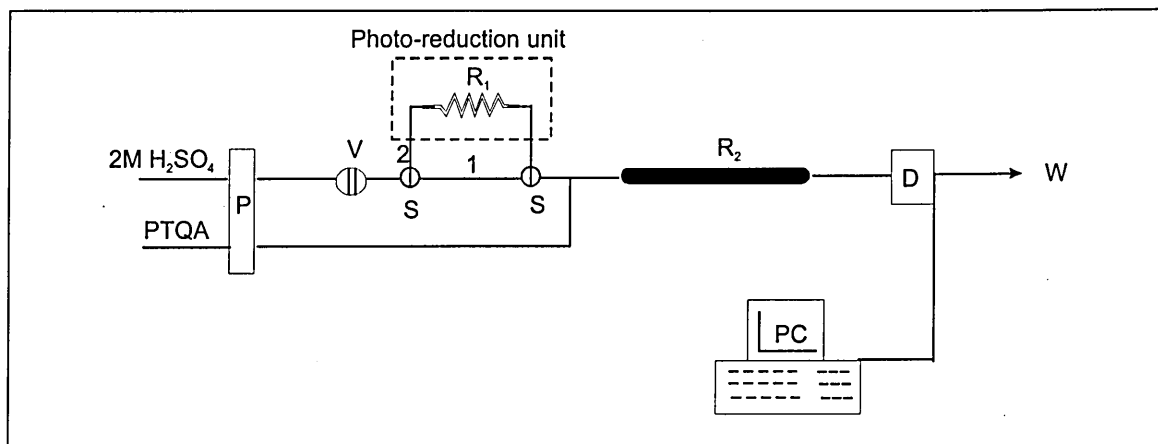


Figure 5. 4 From Ahmed et al (13). Schematic representation of the FI manifold employed for the simultaneous determination of Se^{IV} and Se^{VI} . P, pump; V, valve; S, selector valve; R_1 , photoreduction coil; R_2 , single bead string reactor (SBSR); D, detector; W, waste; and PC, personal computer.

Comparing the systems shown in Figure 5. 2, Figure 5. 3, and Figure 5. 4, it can be seen that the latter is the least complex. The major drawbacks in the use of DAN are that the sample has to be boiled for at least 20 minutes in order to produce the

piazoselenol, and also liquid-liquid extraction of the piazoselenol into either toluene or cyclohexane is required before detection.

In this study, an on-line fluorescence method based on the reaction of Se (IV) with PTQA in acid medium is developed.

5.2 RESULTS AND DISCUSSION

5.2.1 Conditions for Se (IV) determination

5.2.1.1 Flow rate

The system used for the determination of Se (IV) is shown in Figure 5. 5. A 1:1 ratio of acid to PTQA was used in order to simplify the manifold.

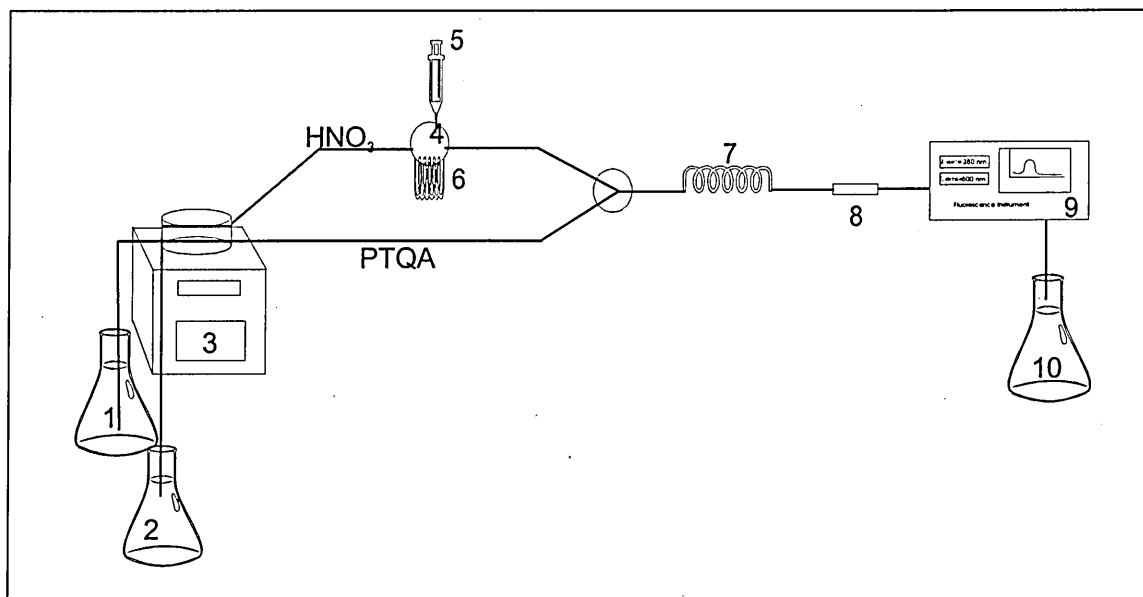


Figure 5. 5 On-Line system for Se (IV) determination. 1 1M HNO₃, 2 5x10⁻⁴ M PTQA solution, 3 peristaltic pump, 4 injection valve, 5 sample in; 6 sample loop; 7 mixing coil; 8 on-line filter; 9 fluorescence detector; 10 waste.

Flow rate was optimised using a 200 µg L⁻¹ of Se (IV), and it was increased from 0.41 to 1.09 mL min⁻¹ by adjusting the pump speed. Using the low flow rate a broad peak was observed but from 0.68 mL min⁻¹ peak shape was sharper. However, when flow rates higher than 0.82 mL min⁻¹ were used, the total peak area decreased (see fig. 5.6). The optimum flow rate was 0.68 mL min⁻¹.

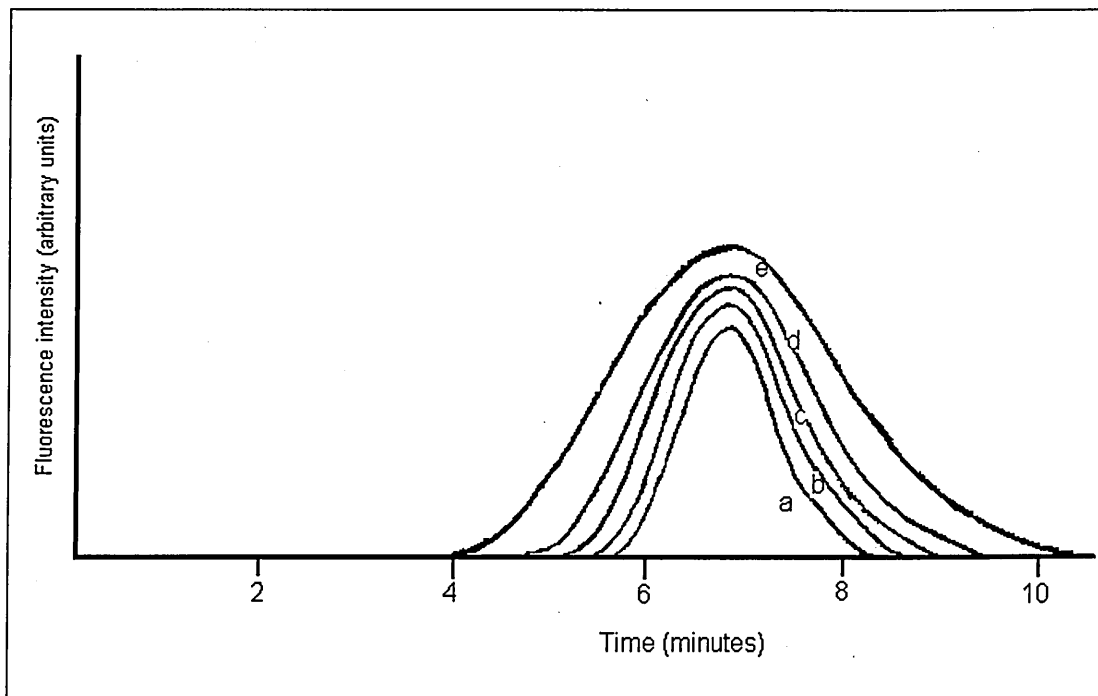


Figure 5. 6 The effect of flow rate on peak shape of the Se (IV) signal generated, using the following condition: $200 \mu\text{g L}^{-1}$, a solution 0.5 M HNO_3 , $5 \times 10^{-4} \text{ M PTQA}$, using tubing of 1.0 mm internal diameter. Flow rate (mL min^{-1}): a: 0.41 , b: 0.54 , c: 0.68 , d: 0.82 , e: 1.09 .

5.2.1.2 The effect of acid on the fluorescence signal intensity

As shown in the off-line method, the most intense signals were obtained using HNO_3 . Fluorescence intensity was found to increase up to 2 M HNO_3 , after which, there was a slight decrease of about 5% when the acid concentration was doubled.

5.2.1.3 Effect of sample loop size on signal shape

Initially, the Se (IV) solution used for these experiments was prepared in deionised water. A single peak was observed when the $500 \mu\text{L}$ sample loop was used. However, with the bigger $1000 \mu\text{L}$ sample loop a double peak was observed, an indication that poor mixing with the 2 M HNO_3 carrier stream was occurring.

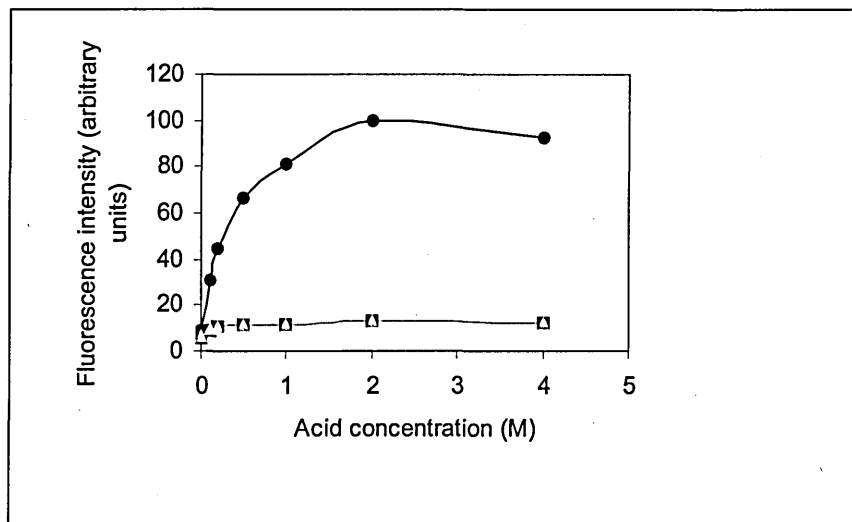


Figure 5.7 Acid influence in the on-line fluorescence reaction. Using the following conditions: a solution of $200 \mu\text{g L}^{-1}$ Se (IV) in acid, flow rate 0.75 mL min^{-1} ; $5 \times 10^{-4} \text{ M}$ PTQA (-x-) HNO_3 , (-■-) H_2SO_4 , (-▲-) H_3PO_4 .

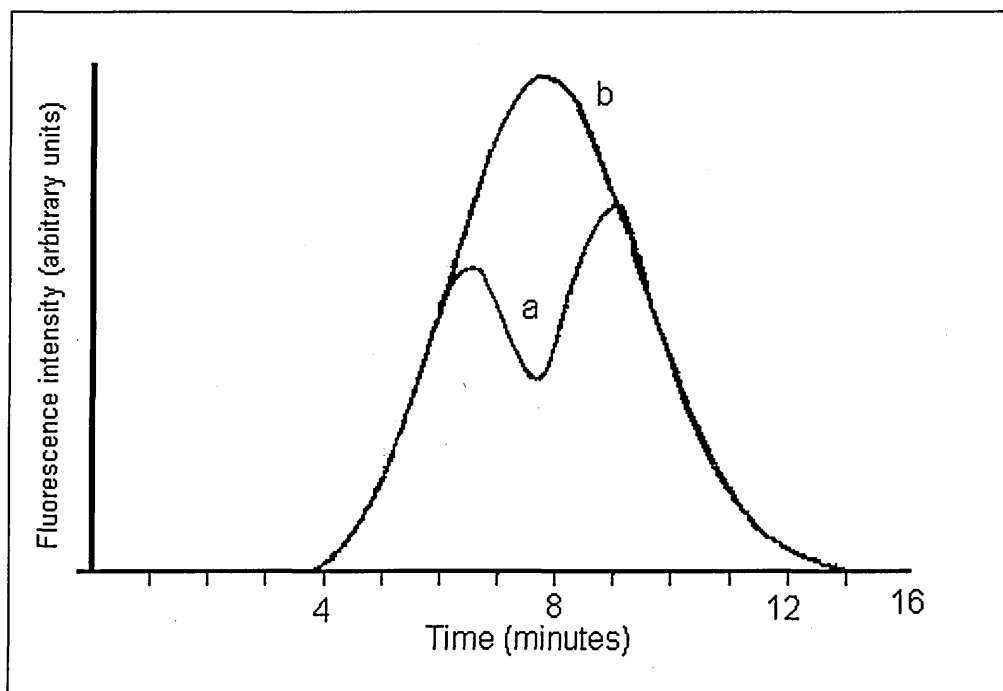


Figure 5.8 Effect of sample loop size on Se (IV) peak shape. Using the following conditions: a solution of $500 \mu\text{g L}^{-1}$ Se (IV) in water, flow rate: 0.75 mL min^{-1} ; $5 \times 10^{-4} \text{ M}$ PTQA; Loop size, a: $1000 \mu\text{L}$ and b: $500 \mu\text{L}$.

When selenium solution was prepared in the carrier solution, and using the bigger sample loop ($1000 \mu\text{L}$) a single intense peak was obtained as shown in Figure 5. 9. Subsequent samples were prepared in carrier solution, and a $1000 \mu\text{L}$ sample loop was used for further selenium determinations.

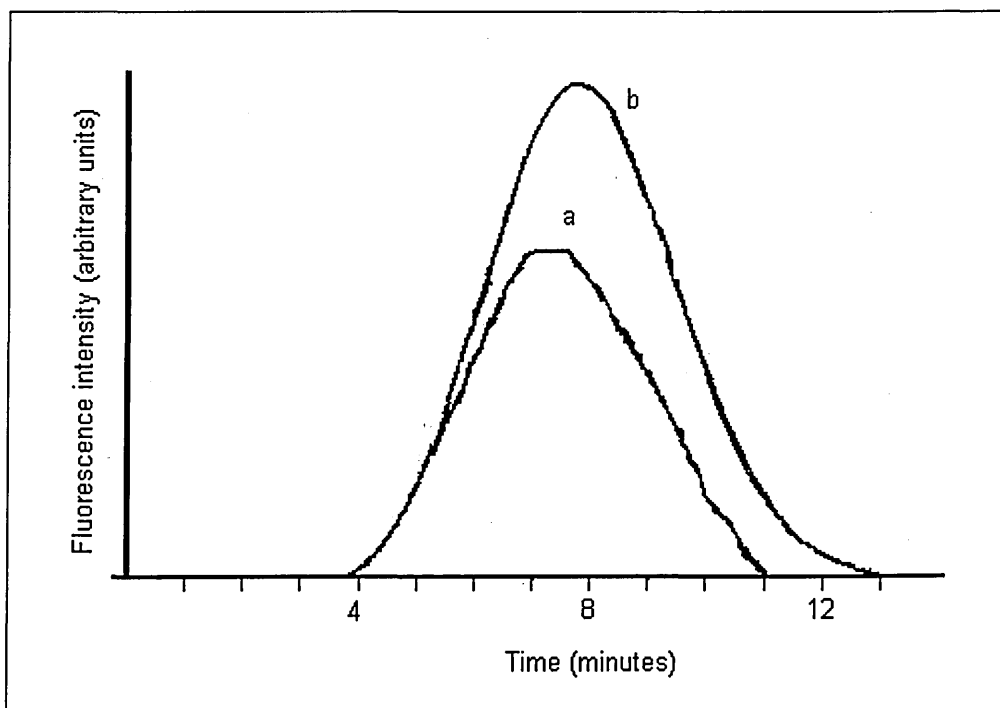


Figure 5. 9 The effect of loop size on peak area, using the following conditions: a solution of $500 \mu\text{L L}^{-1}$ Se (IV); 5×10^{-4} PTQA, 1M HNO_3 ; Loop size, a: $500 \mu\text{L}$, b: $1000 \mu\text{L}$.

5.2.2 Se (IV) and Se (VI) determination after photoreduction

5.2.2.1 Development of the on-line system

As previously discussed, basic conditions are required for the photoreduction of Se (VI) to Se (IV). In contrast, the Se-PTQA reaction must be carried out in acid medium. Consequently, in developing an on-line method, consideration has to be given to the means of changing the condition from very basic, 5 M NaOH, to very acid, 2M HNO_3 . In order to neutralise the basic solution a high concentration of acid is required. Attempts to neutralise the sodium hydroxide with acid concentrations of 6M and 4M HNO_3 , resulted in the formation of bubbles, which caused the instrument baseline to drift. The acid concentrations were reduced but the proportion in the system was increased by changing the rate at which it was pumped and mixed with sodium hydroxide. Using a NaOH/ HNO_3 pump ratio of 0.30/0.75, it was possible to use 2.5 M HNO_3 for neutralisation. A very stable baseline and satisfactory conversion of Se (VI) to Se (IV) were obtained.

5.2.2.2 Optimisation of photoreactor size

The extent of Se (VI) conversion to Se (IV) by photoreduction is determined by the length of time the sample is exposed to UV radiation. In the on-line system, the exposure time is determined by the reactor coil length and sample flow rate. Initially, a 10.75 m length reactor and 0.8 mm internal diameter coil was investigated. To increase the irradiation time, flow rates of 0.2 and 0.3 mL min⁻¹ were used. A broad band was obtained at the longer reactor coil length, and as a result, shorter reaction coil lengths of 5.25 and 2.50 m were investigated. A comparison of the effect of shortening the reaction coil length is shown in Figure 5. 10. As expected the extent of Se (VI) conversion to Se (IV) as measured by the area under the peak is reduced. The effect of reduced flow rate from 0.3 to 0.2 mL min⁻¹ is shown in Figure 5. 11. Although the peak area at 0.2 mL min⁻¹ was increased, the peak was very broad. In practice, this will lead to lengthy analysis time. In order to reduce the amount of reagent used and sample dispersion, stopped flow analysis with a shorter coil length (2.5 m) was investigated.

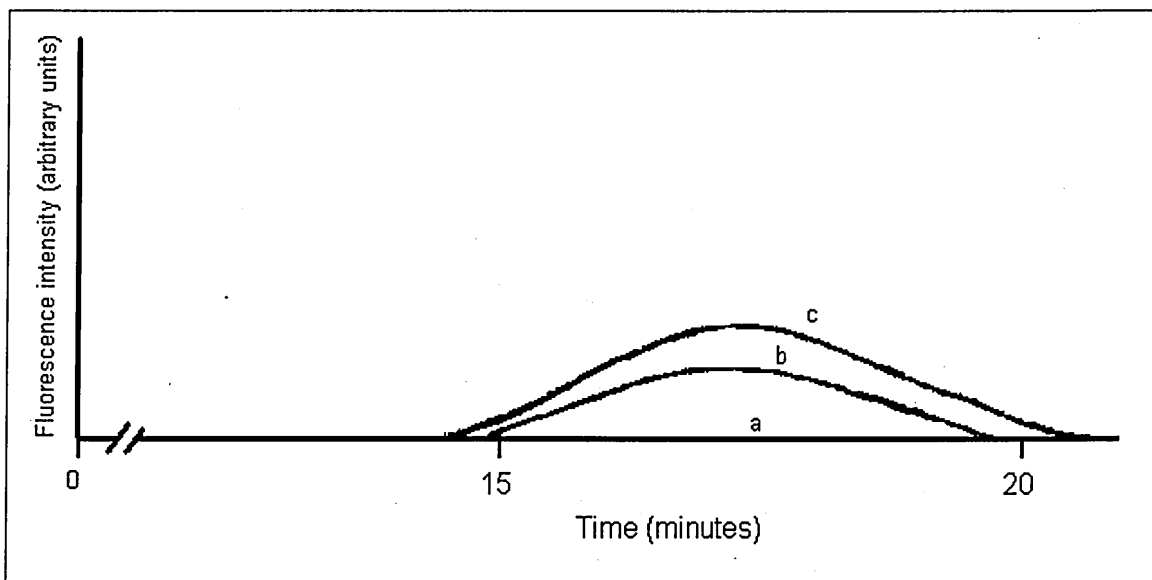


Figure 5. 10 Comparison of the effect of shortening the reaction coil length, using the following conditions: a solution of 200 $\mu\text{g L}^{-1}$ Se (VI) in 5 M NaOH (0.30 mL min⁻¹) and 2.5 M HNO₃ (0.75 mL min⁻¹), coil length (m): a: 2.50, b: 5.25 and c: 10.75.

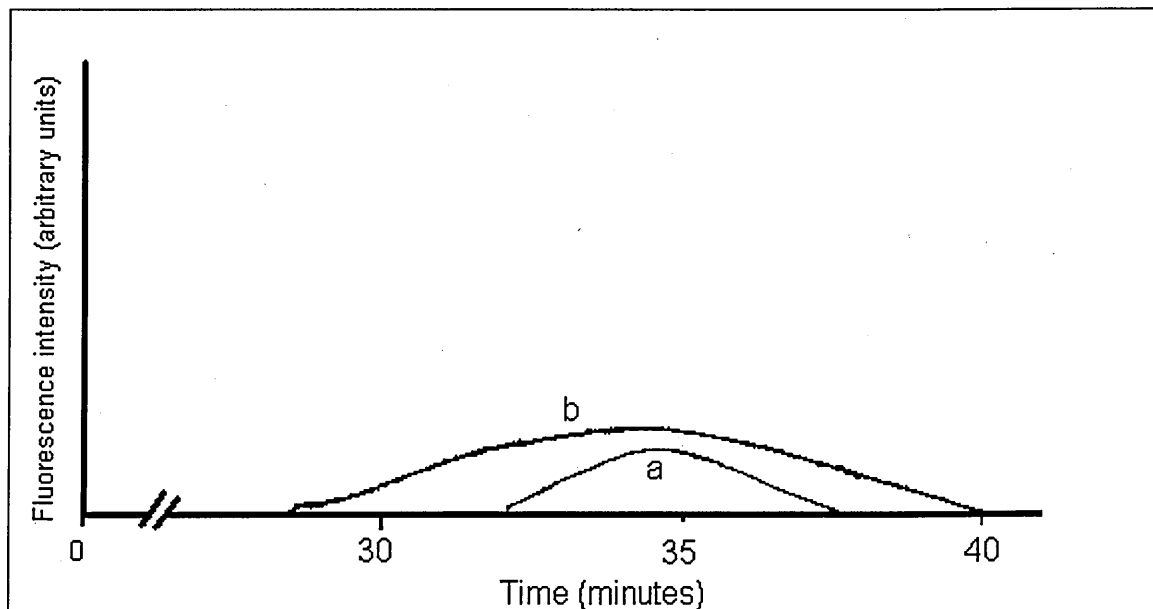


Figure 5. 11 Reduction of NaOH flow rate for increasing of photoreduction, using the following conditions: a solution of $200 \mu\text{g L}^{-1}$ Se (VI); photo coil: 10.50 m; 5×10^{-4} M PTQA; 2.5 M HNO_3 ; HNO_3 flow rate: 0.75 mL min^{-1} ; NaOH flow rate a: 0.3 mL min^{-1} ; b: 0.2 mL min^{-1} .

5.2.2.3 Stopped flow time

The system used for the simultaneous analysis of Se (IV) and Se (VI) with stopped flow is shown in Figure 2.6 (see chapter 2, section 2.3.4.2). In the off-line method, 40 minutes was found to be optimum for the photoreduction Se (VI) to Se (IV). Therefore, irradiation times between 10 and 50 minutes were studied. As shown in Figure 5. 12, the most intense signal was obtained when flow is stopped for 50 minutes. No improvement was observed after 50 min. Furthermore, the results obtained at 40 min were comparable with those at 50 min. Improvement in signal dispersion was obtained when the reactor coil length was reduced from 10.75 to 2.5 m., and a more intense signal was observed when flow is stopped.

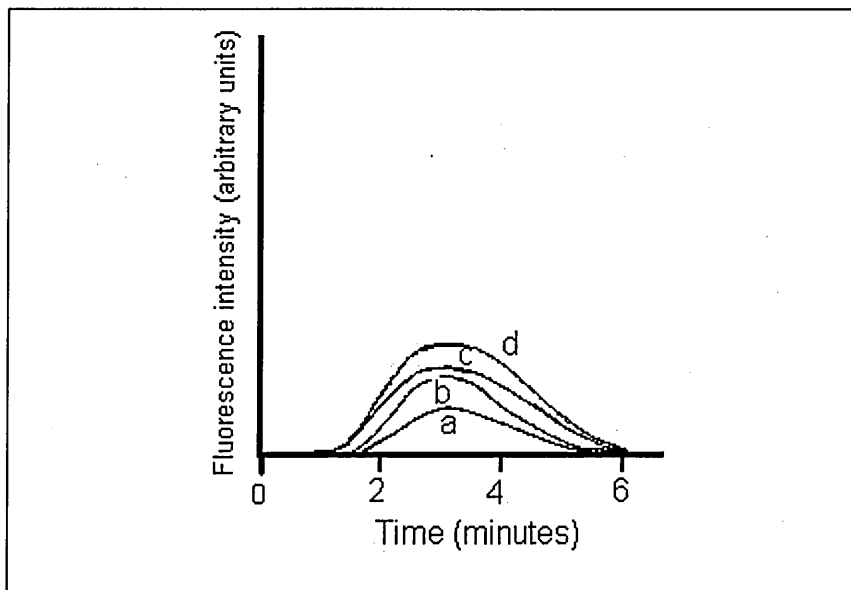


Figure 5. 12 Optimisation the time for the stop flow method for the photo-reduction.
 Using the following conditions: $200 \mu\text{g L}^{-1}$ Se (VI); photo-reactor coil length: 10.50 m;
 5×10^{-4} M PTQA; 2.5 M HNO_3 ; HNO_3 flow rate: 0.75 mL min^{-1} ; NaOH flow rate 0.3 mL min^{-1} ;
 stopped time a: 10 min.; b: 20 min.; c: 30 min.; d: 50 min.

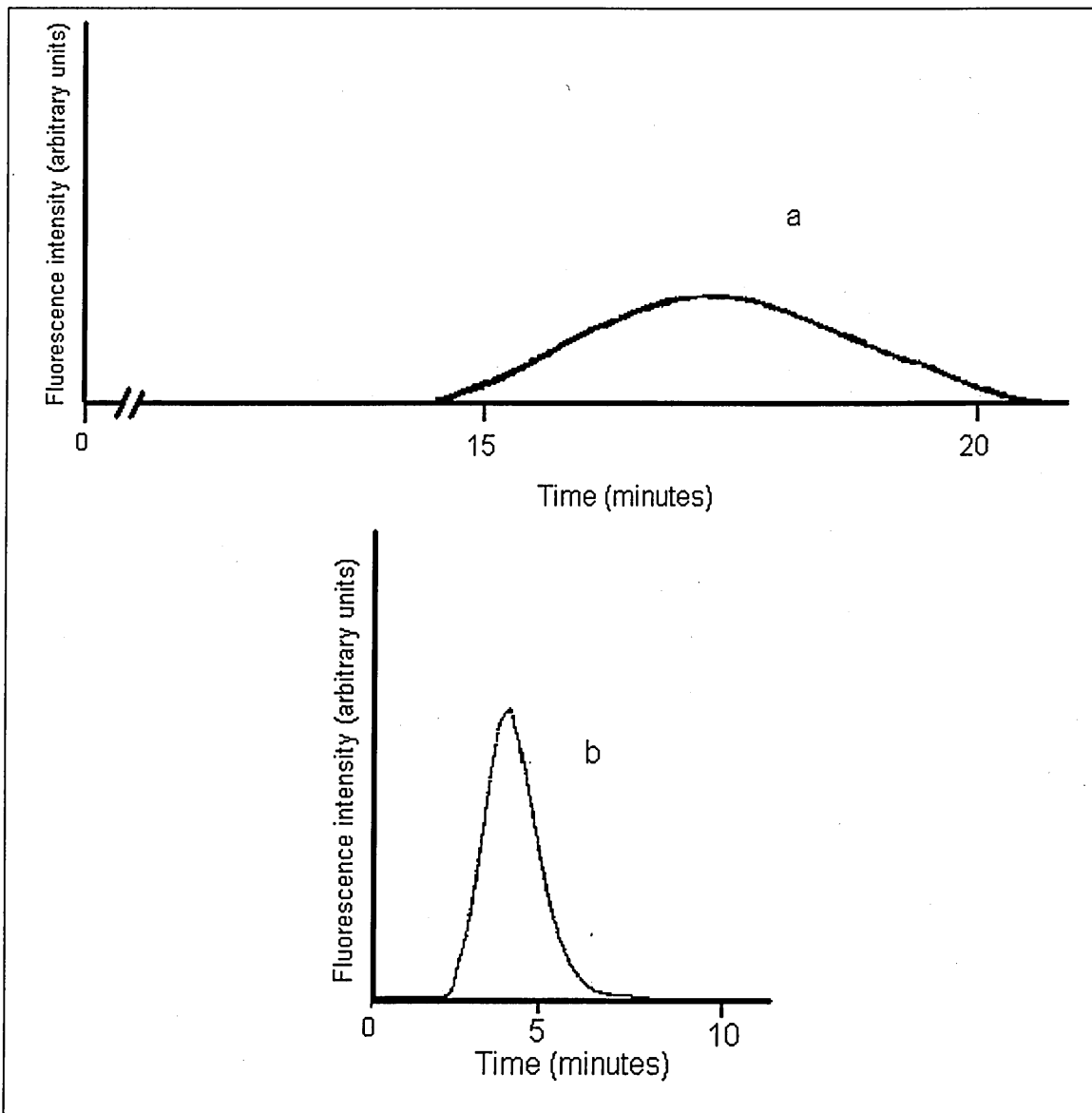


Figure 5. 13 Comparisons between stopped flow and continuous system using the following conditions: $200 \mu\text{g L}^{-1}$ Se (VI); 5×10^{-4} M PTQA; 2.5 M HNO_3 ; HNO_3 flow rate: 0.75 mL min^{-1} ; NaOH flow rate 0.3 mL min^{-1} ; a: continuous system with 10.75 m of coil length, b: stopped flow for 40 minutes, with 2.5 m coil length.

5.2.2.4 Calibration graph and repeatability

Figure 5. 14 shows the signals obtained when a solution of $200 \mu\text{g L}^{-1}$ Se (IV) is injected four times into the on-line system. For the measurements at this level the coefficient of variation was 2.7 %. The determination took about 28 minutes, which corresponds to a throughput of 8 samples/hour. The detection limits of this method defined as 3σ of the blanks was $40.0 \mu\text{g L}^{-1}$ compared to $0.4 \mu\text{g L}^{-1}$ determined for the batch procedure. The difference in the sensitivities of the detectors could account for the differences in the calculated detection limits. This reduction in the detection limit could be due because HNO_3 concentration, as well as, the

fluorescence detector used for the on-line method. The calibration graph in the concentration range 40-500 $\mu\text{g L}^{-1}$ is shown in Figure 5. 15.

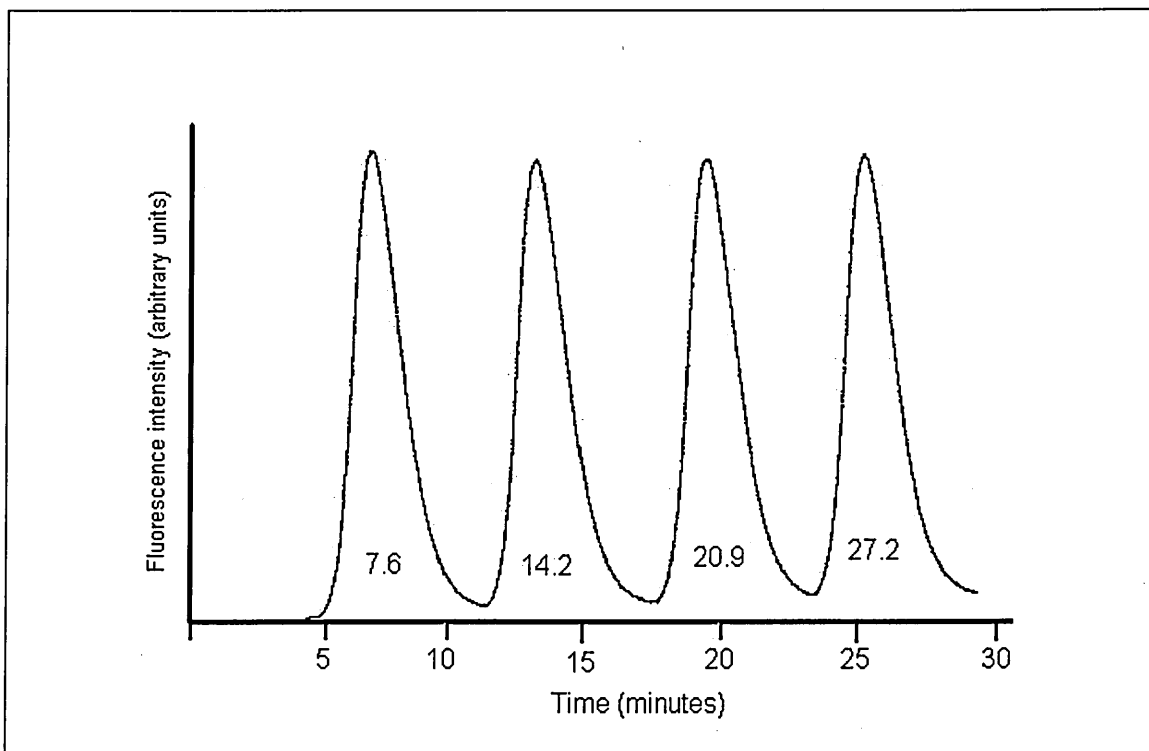


Figure 5. 14 Repeatability for four Se (IV) injections at 200 $\mu\text{g L}^{-1}$ Se (IV); photo reactor coil length: 2.50 meters; 5×10^{-4} M PTQA; 2.5 M HNO_3 ; HNO_3 flow rate: 0.75 mL min^{-1} ; NaOH flow rate 0.3 mL min^{-1} ; stopped flow for 40 min.

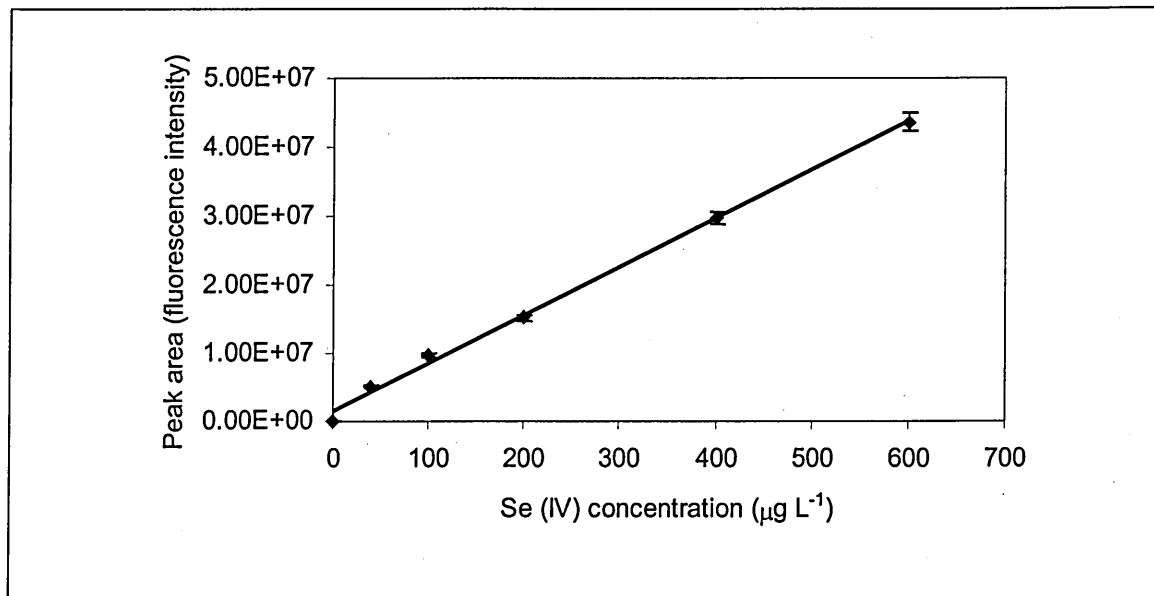


Figure 5. 15 Calibration graph for Se (IV) in the concentration 40 to 500 $\mu\text{g L}^{-1}$ Se (VI); photoreactor coil length: 2.50 meters; operating conditions 5×10^{-4} M PTQA; 2.5 M HNO_3 ; HNO_3 flow rate: 0.75 mL min^{-1} ; NaOH flow rate 0.3 mL min^{-1} . $y = 70427x + 2\text{E}+06$; $r^2 = 0.9966$.

5.2.2.5 Application of the on-line method to sample analysis

The developed method was applied to five different selenium supplements. Samples were injected with the UV-lamp turned off to determine the Se (IV) content. A second injection into the system, but this time with the lamp turned on, was used for the determination of total selenium. The Se (VI) content was determined by difference. Figure 5. 16 shows the peaks obtained from the determination of Se (IV) and total selenium before and after UV-irradiation, and 40 min stopped flow in a sample. Recoveries from this sample were between 90-98 %. Lower recoveries (50-60 %) were found for samples A, B and D. Initially it was thought that the low recovery was as a result of selenium being the organic form. However, when selenium speciation was determined by the ICP-MS method (see Chapter 6), it was shown that sample C, which gave the highest recovery, contained selenomethionine, an indication that organoselenium reacts with PTQA in acid medium. Analysis of selenocystine and selenomethionine using the batch method (Chapter 4) showed that the organic form of selenium produced between 50-60 % of the signal expected for selenite (Table 5. 1). These low recoveries are due to extraction problems, because using the ICP-MS method, it was found that higher recoveries were achieved using shaking extraction.

Table 5. 1 Selenium determination as Se (IV) in selenomethionine and selenocystine solutions using the batch fluorescence method (Chapter 4).

Se concentration ($\mu\text{g L}^{-1}$)		
Sample	Expected	Found
Selenomethionine	100	48.8 ± 2.2
Selenocystine	100	43.4 ± 1.8

As discussed in chapter 6, it was found that ultrasonic extraction is less effective for extraction of selenium species in the supplements when compared to shaking. Hence the recoveries observed here. Results for the analysed samples are presented in Table 5. 2.

Table 5. 2 Comparison of the results (n=5) of selenium determination by the developed on-line method with ICP-MS, against expected values in $\mu\text{g L}^{-1}$.

Sample	Se concentration ($\mu\text{g L}^{-1}$)		
	Expected values	ICP-MS method	On-Line Fluorescence method
A	200.0	201.1 \pm 1.5	128.6 \pm 7.8
B	25.0	25.4 \pm 1.1	17.9 \pm 2.2
C	25.0	25.8 \pm 1.7	24.8 \pm 1.0
D	100.0	98.4 \pm 2.0	59.0 \pm 4.3
E	50.0	48.9 \pm 1.8	51.0 \pm 1.2

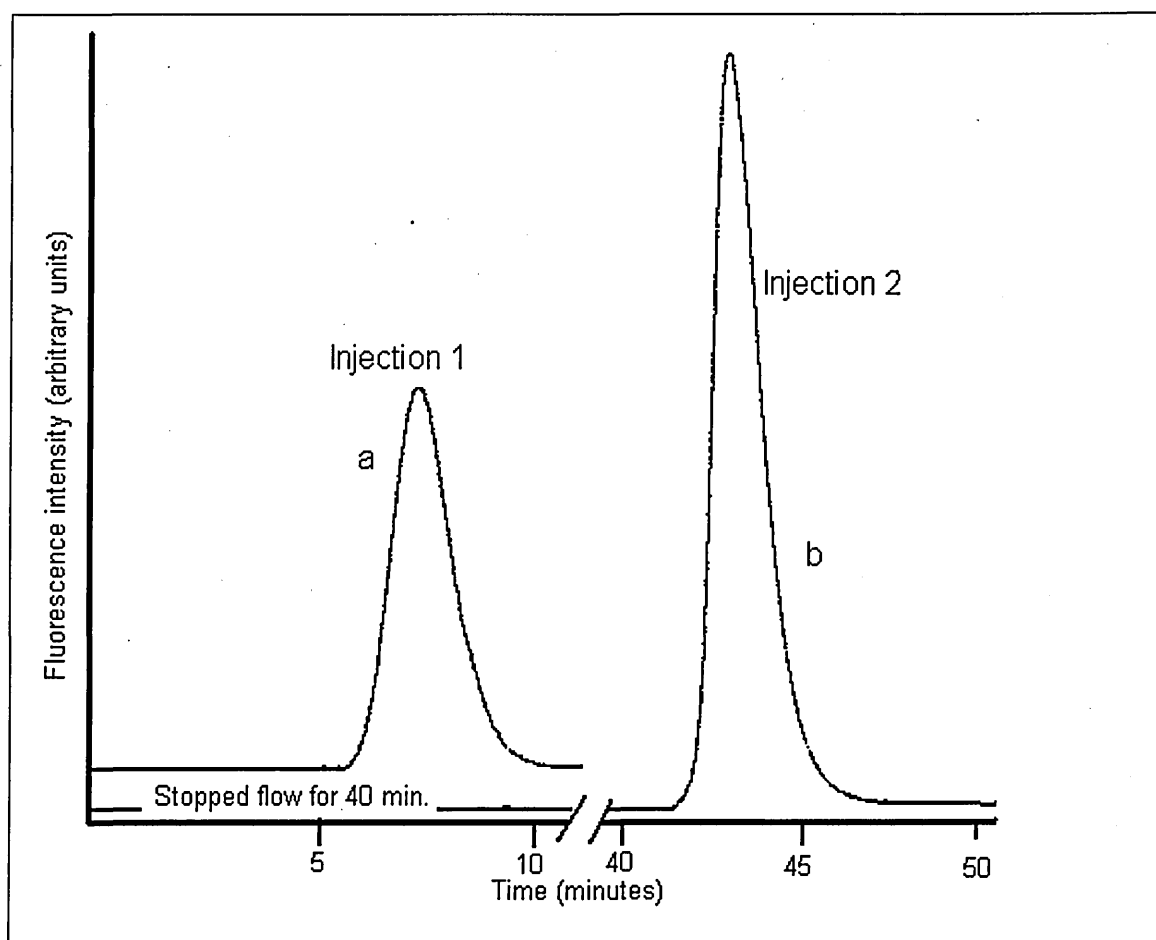


Figure 5. 16 Sample C analyses, using the following conditions, solution of $200 \mu\text{g L}^{-1}$ Se (VI); photoreactor coil length: 2.50 meters; 5×10^{-4} M PTQA; 2.5 M HNO_3 ; HNO_3 flow rate: 0.75 mL min^{-1} ; 5 M NaOH flow rate 0.3 mL min^{-1} ; stopped flow for 40 min; a: injection and direct determination of Se (IV); b: injection and sample irradiation for 40 minutes and detection as total selenium (selenite and selenate).

5.3 CONCLUSION

The use of an on-line photoreduction reactor in a basic medium, (5 M NaOH) was shown to be effective for the reduction of Se (VI) to Se (IV) with a stopped flow system, over 40 minutes, at 80 ° C.

Automation of the system resulted in much shorter analysis times, (eight samples per hour), for Se (IV) and 45 minutes for Se (VI). Simultaneously five samples of selenite could be analysed at the same time that one selenate samples is irradiated. This method was very sensitive, especially for Se (IV) where a direct reaction with PTQA is involved.

The extraction method used was effective for liquid samples (98 % recovery) but it does need improve for the solid samples because recoveries of only 55 % were achieved.

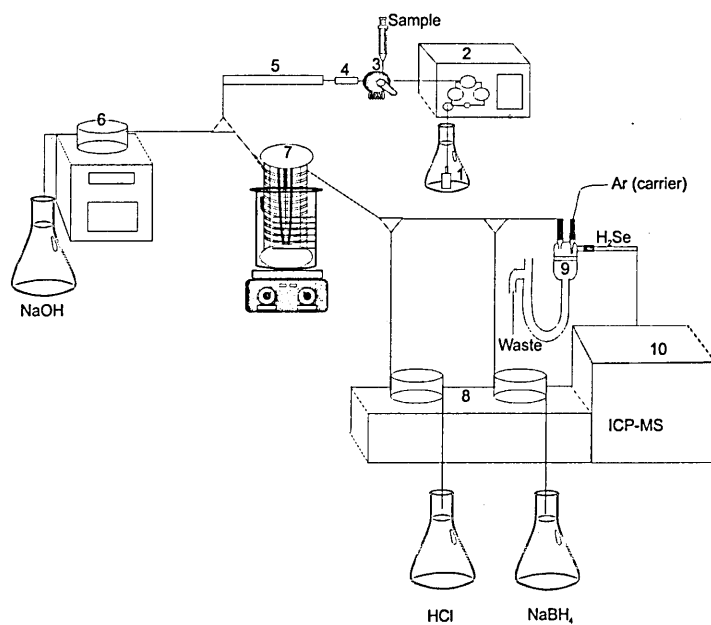
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Chapter 6:

Selenium Speciation in Nutritional Supplements using HPLC-UV irradiation with HG-ICP-MS Detection



6.1 INTRODUCTION

Over the last five years, the increased interest in selenium has been matched by the development of methods for its determination in a wide variety of sample types. The focus of more recent research has been the study of the various forms of the element in environmental and biological samples. Identifying the chemical forms, chemical speciation, enables predictions to be made about the likely chemical and biological behaviour of the element. The distribution and type of selenium-containing species in food and food supplements determine the bioavailability of the element. Therefore the development of robust and reliable methods for the study of the distribution of selenium species is essential for a complete understanding of selenium assimilation in the human body.

Chemical speciation involves separation of the various chemical forms followed by partial or complete identification of the species. One of the earliest separation methods was precipitation of the selenium-containing compound. The differences in the adsorption characteristics of the selenium forms on silica, alumina (1-3) and other ion-exchange-resins (4-7) have been exploited in order to achieve separation of the individual species. Sequential extraction with the use of acid, basic or organic solvent is also widely applied for selenium-speciation (8 - 12). Less commonly used is enzymatic extraction (13, 14). On the other hand, capillary electrophoresis (CE) is a technique that is in increasing use for the separation of selenium forms (15 - 22) but is still a problem with regard to the stability of the capillary as well as the detection system. The most commonly used techniques involve chromatography. Gas chromatography (GC) is used for separation of volatile species of selenium, but volatility is a limitation (23 - 29). HPLC is the most popular because of its versatility. The big difference between GC and HPLC is that with HPLC, high molecular weight structures such as proteins can be separated without previous derivatisation. HPLC may also separate inorganic and organic selenium forms in the same run. HPLC has been coupled to a range of detector-systems, such as electro-thermal atomic absorption spectrometry (ETAAS) (30-33), atomic absorption spectrometry (AAS) (34-39), atomic fluorescence spectrometry (AFS) (40-43), and also to inductively coupled plasma (ICP) with atomic emission spectrometry (ICP- AES) (32) or mass spectrometry (ICP-MS) (4, 44 - 71). As shown in Figure 6. 1, coupled LC-ICP-MS accounts for more than 60 % of the total number of applications. It is noteworthy that the different HPLC modes, i.e. reversed phase (RP), ion pair (IP), ion exchange (IE) and size exclusion (SE), have all been used for the separation of selenium species. RP chromatography is preferred when predominately non-polar species are

separated. IE chromatography offers better resolution for ionic selenium species. The application of the latter is simple and cheaper (72). Since the concentrations of the selenium species in the column fractions are usually less than $1 \mu\text{g mL}^{-1}$, it is essential that the detector selected is sensitive. Inductively coupled plasma mass spectrometry (ICP-MS) is a technique that can be used to detect selenium at levels less than $1 \mu\text{g L}^{-1}$ in solution. Better precision and sensitivity are achieved for elements such as Se, Te, Sn, Pb and Ge when they are converted into hydrides before detection. In addition, matrix interferences are reduced and also possible blockage of the sampling cone caused by salt deposits from the column eluent is eliminated when hydride generation is used (36, 37, 73, 74). However, before the hydride is formed, all other selenium species in oxidation states other than (IV) must either be oxidised or reduced (36, 75, 76). Consequently, on-line sample pretreatment, which involves reduction/oxidation to produce Se (IV) and its transformation to hydrogen selenide, is required.

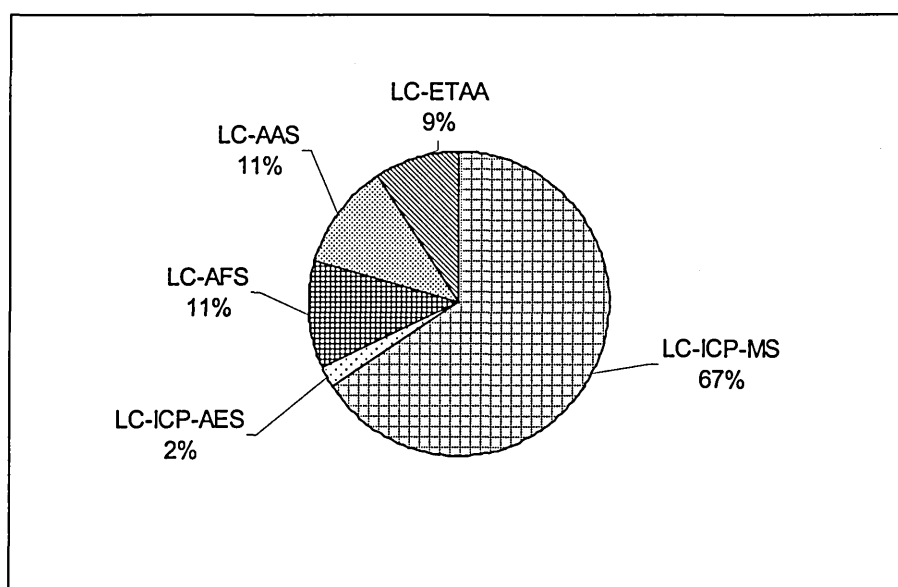


Figure 6. 1 Detectors used for selenium speciation by LC.

Using an ion-exchange column and a phosphate buffer mobile phase, Gomez et al. (34) determined trimethylselenonium (TMSe^+), selenomethionine (SeMet), and selenocysteine (SeCys) in urine. The column eluent was mixed with a solution of 3% $\text{K}_2\text{S}_2\text{O}_8$ and 3 % NaOH on-line in order to oxidise the organic Se species to selenate followed by reduction to selenite in 10 M HCl . Hydride generation was achieved in a solution of 0.5 % NaBH_4 in 0.16% NaOH . González et al (35) separated selenoethionine (SeEt), selenomethionine (SeMet), selenite (Se, IV) and selenate (Se, VI) in urine and water using an anion-exchange column. After conversion to selenite using a mixture of HBr/KBrO_3 with microwave assisted heating each

species was then converted to the hydride before detection with either ICP-AES and ICP-MS. The detection limits obtained by ICP-MS for inorganic selenium, Se-Met and Se-Et were 0.16, 0.59, and 0.66 $\mu\text{g L}^{-1}$, respectively. In subsequent work by Gómez et al. (7), in which, SeCys, SeMet, SeEt, Se (IV) and Se (VI) were separated using RF and IE, followed by HBr/KBrO₃ (47%/15 mM) oxidation, the detection limits reported on the five species were 0.6, 0.8, 0.9, 0.8 and 0.6 $\mu\text{g L}^{-1}$, respectively. Reverse phase chromatography has been applied to the separation of selenocysteine (SeCys), selenomethionine (SeMet), selenoethionine (SeEt) and selenite on a C₁₈ column with ammonium acetate buffer solution containing 0.5 % methanol and 10⁻⁵ mol L⁻¹ di-(dodecyl)-methylammonium bromide as mobile phase. The detection limits obtained were 18, 70, 96, and 16 $\mu\text{g L}^{-1}$, respectively for the atomic fluorescence spectrometry technique used (40). Vilanó et al. (38), developed an on-line UV irradiation method for the reduction of selenate to selenite. In subsequent work by the same group (77), both high and low pressure mercury lamps with power ratings of 150 W and 15 W, respectively were used for the photoreduction of selenate to selenite. The selenium atomic fluorescence signals obtained from the latter were thrice those from the former. The lower efficiency of the high pressure lamp was put down to reoxidation of selenite to selenate. Detection limits for Se (VI), SeCys and SeMet were 0.6, 14.5, 0.9 and 5.9 $\mu\text{g L}^{-1}$, respectively.

In the work described in this chapter, selenomethionine, selenite and selenate were separated on an ion-exchange column using as mobile phase NaHCO₃/Na₂CO₃ (3.5 M). A hybrid system of UVred- HG-ICP-MS was used for sample pretreatment, and finally the developed method was applied to the analysis of three selenium supplement samples.

6.2 RESULTS AND DISCUSSION

6.2.1 Conditions for the generation of hydrogen selenide (H₂Se)

Initial experiments with each selenium species showed that, in the basic column eluent, no signals for any of the species were detected using the system shown in Figure 6. 2. Signals for selenomethionine and selenite were obtained when 2M HCl was added before hydride generation using the ICP-MS peristaltic pump and a small mixing coil before the addition of NaBH₄. The signals obtained for both species are shown in Figure 6. 3

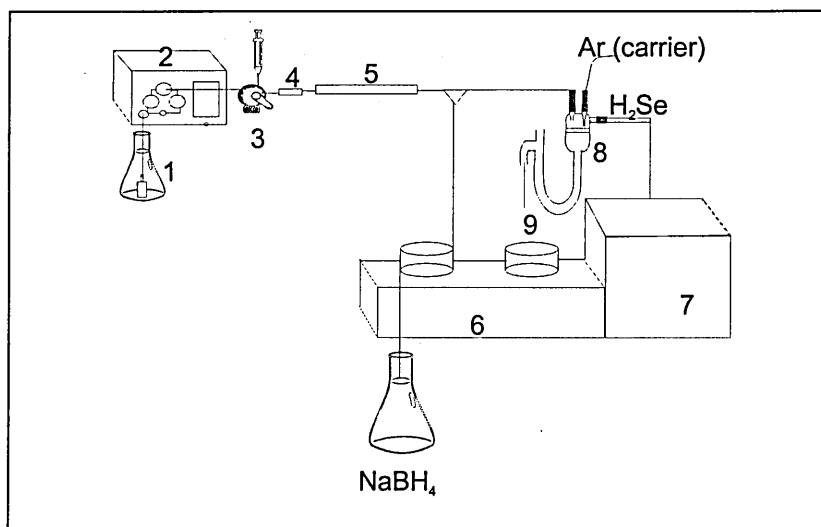


Figure 6. 2 Initial on-line system. 1 mobile phase, 2 HPLC pump (0.7 mL min^{-1}), 3 Inject valve with loop of $50 \text{ }\mu\text{L}$, 4: guard column, 5 Dionex ion-exchange AS9-SC 4 mm column, 6 ICP-MS pump (0.8 mL min^{-1}), 7 ICP-MS instrument, 8 Gas-liquid separator unit.

No signals for selenate were observed in acid concentrations of 12 M HCl without heating the column eluent. As shown in Figure 6. 4, less than 10% conversion of selenate was observed after heating the mixture in a reaction coil to 100°C in a water bath.

It was clear that heating alone was ineffective in converting all of the selenate to selenite. When hydrochloric acid was substituted for hydrobromic acid, a stronger reducing agent, no improvement in the selenate signal was observed. However a mixture of HBr and KBrO_3 with microwave assisted heating (34), in which selenate is reduced to selenite, was not investigated. In order to avoid the use of a mixture of reagents, the use of photoreduction, shown to be effective by other workers (38, 77) was investigated. The action of NaOH together with UV irradiation were investigated because a preliminary study, in which molecular fluorescence was used to detect the presence of selenite, showed that selenate can be reduced to selenite in basic medium in the presence of UV light.

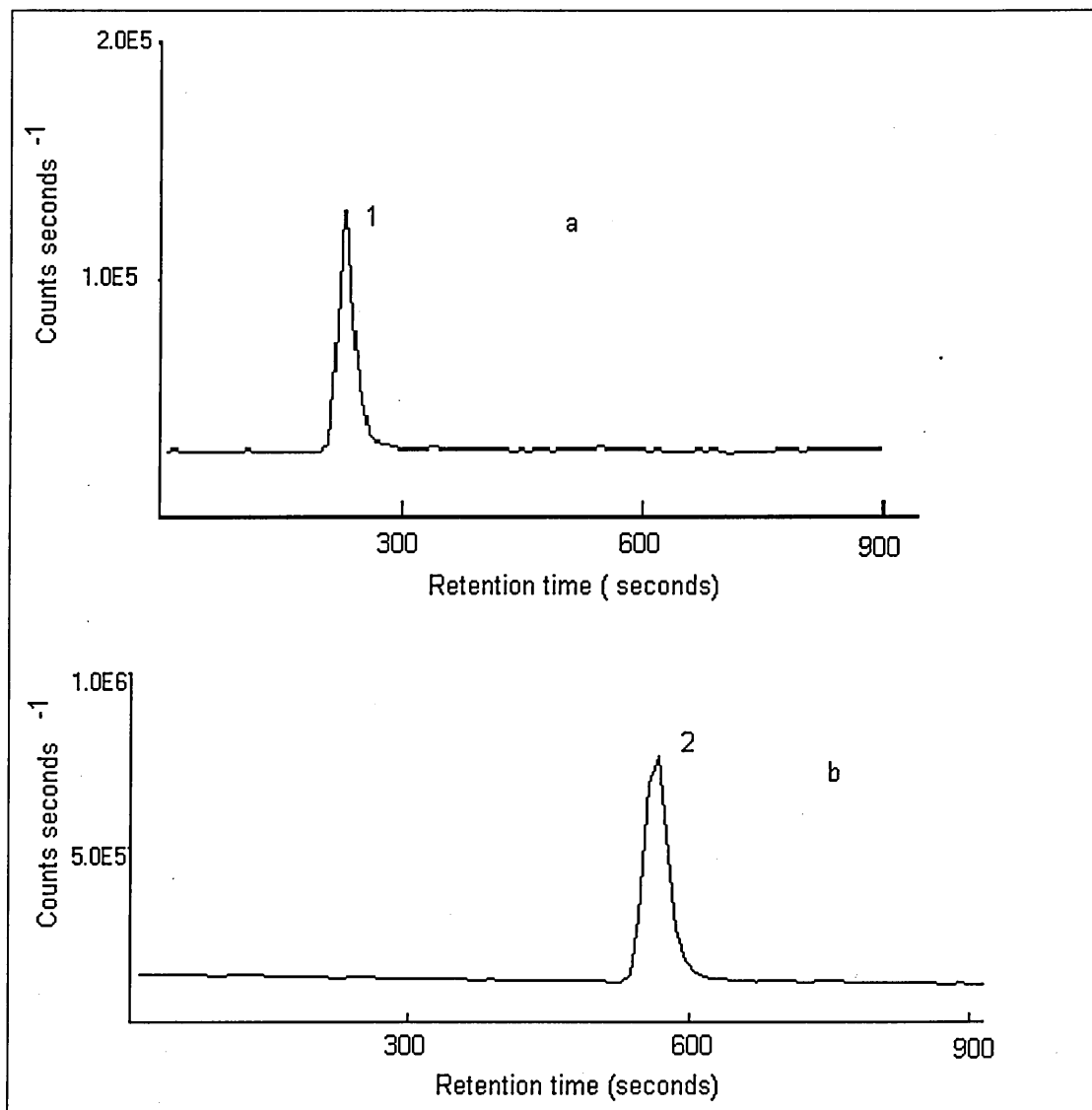


Figure 6. 3 Borohydride reaction products for selenomethionine (a) and selenite (b); Dionex ion-exchange AS9-SC 4 mm column, mobile phase: $\text{Na}_2\text{CO}_3/\text{NaHCO}_3$ 1.8/1.7 (0.5 mL min^{-1}); 2 M HCl and 1% NaBH_4 in 0.1 M NaOH (0.8 mL min^{-1}).

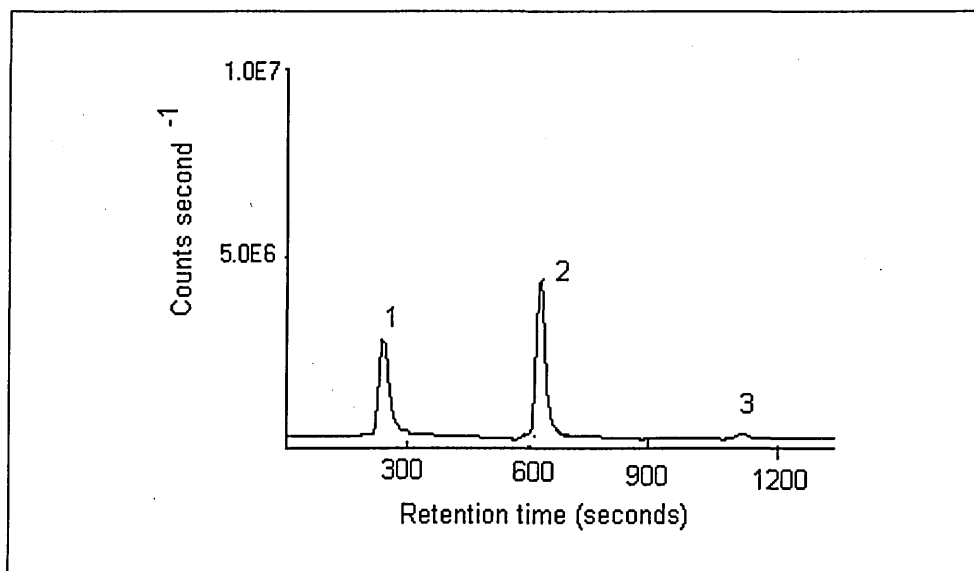


Figure 6. 4 Effect of HCl concentration increased for the H_2Se generation from each selenium form. Column: ion - exchange AS9-SC 4 mm, Dionex, mobile phase: $\text{Na}_2\text{CO}_3/\text{NaHCO}_3$ 1.8/1.7 (0.5 mL min^{-1}); 12 M HCl and 1% NaBH_4 in 0.1 M NaOH (0.8 mL min^{-1}), 100°C .

However, when this experiment was replicated using the system shown in Figure 6. 5.a, no hydride signals for any selenium form were detected. Addition of 4 M HCl to the photoreactor stream (see Figure 6. 5.b) resulted in small signals for the three selenium species. The selenate signal was much smaller than for the other two selenium species. As the amount of acid was increased the signals also increased as shown in Figure 6. 6. The selenate and selenite signals were comparable when the acid concentration was 8.4M, indicating total conversion of selenate to selenite. The effect of increased HCl concentration from 6 to 8.4M was marked for selenate compared with the other two compounds. These results show that the presence of acid is not only essential for the formation of the hydride but also necessary for the reduction of selenate to selenite. All the essayed condition are summarised in Table 6.1.

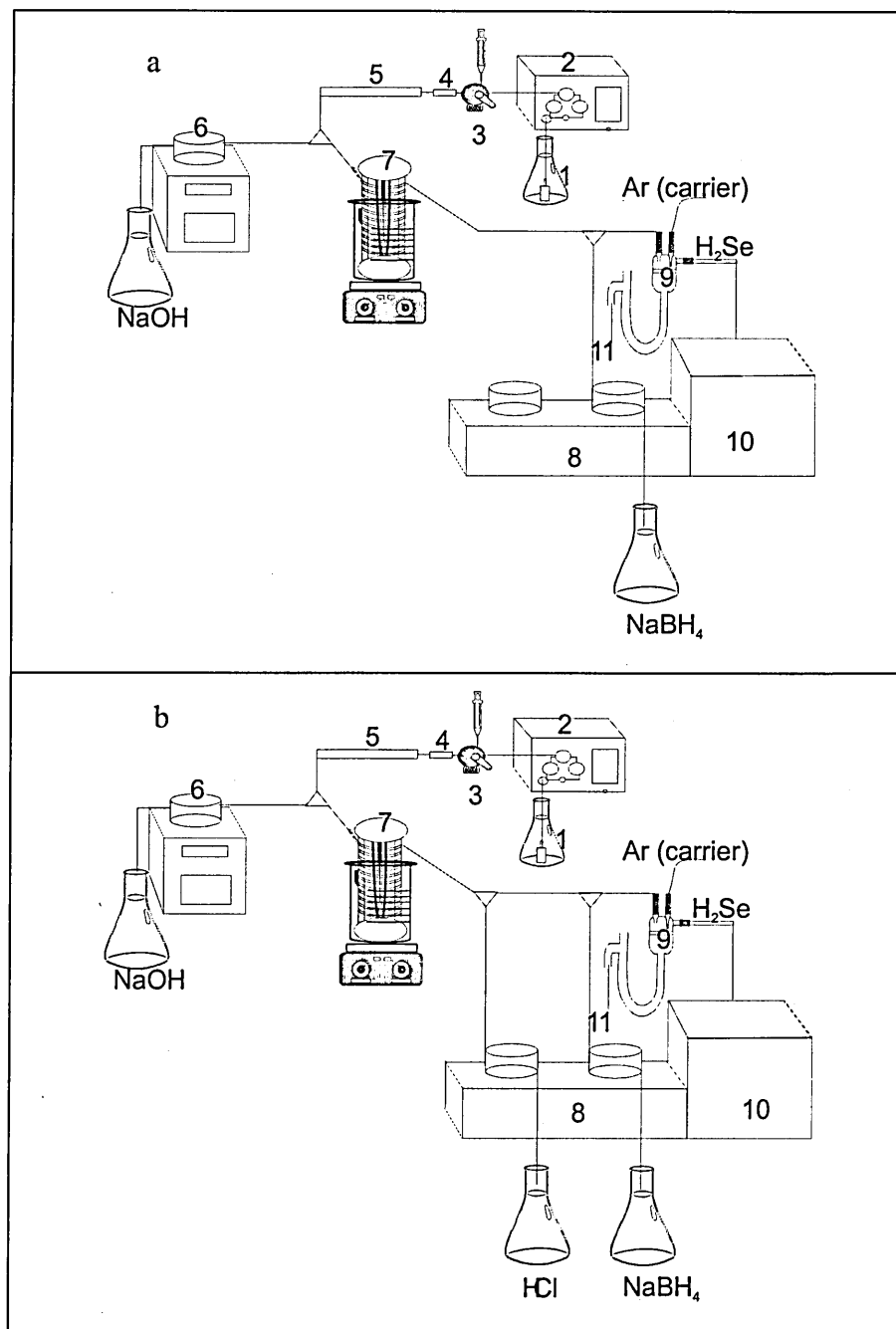


Figure 6. 5 System for on-line speciation of Se by ion-HPLC with post column UV-irradiation (photo-reduction), Hydride Generation and ICP-MS detection. a) without acid and b) with acid addition. 1 mobile phase, 2 HPLC pump (0.7 mL min^{-1}), 3 inject valve with loop of $50 \text{ }\mu\text{L}$, 4 guard column, 5 Dionex ion-exchange AS9-SC 4 mm column, 6 peristaltic pump (0.6 mL min^{-1}), 7 photoreactor, 8 ICP-MS pump (0.8 mL min^{-1}), 9 gas-liquid separator unit, 10 ICP-MS instruments, 11 waste (liquid fraction).

Figure 6. 7 shows the proposed mechanisms for the formation of hydrogen selenide from the separated selenium species. Selenomethionine undergoes reductive deselenization in the presence of sodium borohydride to form hydrogen selenide. Similarly selenite reacts with sodium borohydride under acidic conditions to form the hydride. Although there is evidence to suggest that selenate is reduced in basic

medium in the presence of UV light (see chapter 4), addition of excess HCl acid drove the reaction to completion (38, 40, 77).

6.2.2 The effect of carrier gas flow

Argon gas was used to transfer the hydride from the gas-liquid separator into the plasma. Optimum carrier gas flow is essential as this affects the measurement precision (78). Furthermore, the selenium peak intensity is determined by the rate at which the hydride is transferred into the plasma. When no carrier gas was used (Figure 6. 8) the peak intensity decreased notably, and the sensitivity was much reduced because only a small quantity of the H_2Se formed reached the plasma.

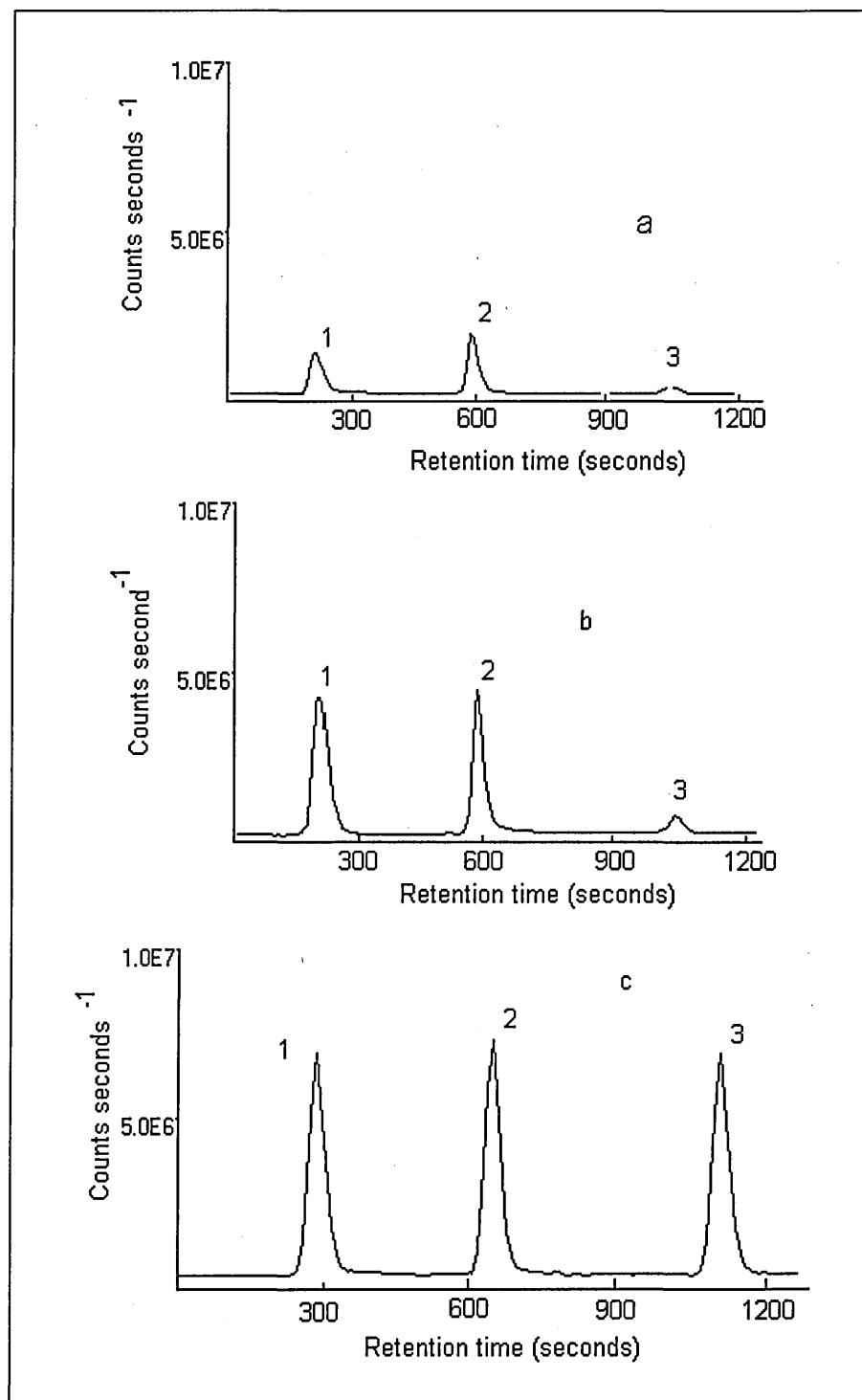


Figure 6. 6 Effect of increased HCl concentration. Condition: Dionex ion-exchange AS9-SC 4 mm column, mobile phase $\text{Na}_2\text{CO}_3/\text{NaHCO}_3$ 1.8/1.7 (0.7 mL min^{-1}); NaOH (0.6 mL min^{-1}), HCl and 1% NaBH_4 in 0.1 M NaOH (0.8 mL min^{-1}). a NaOH 5M and HCl 4 M; b 5 M NaOH and 6 M HCl and c 5 M NaOH and 8.4 M HCl.

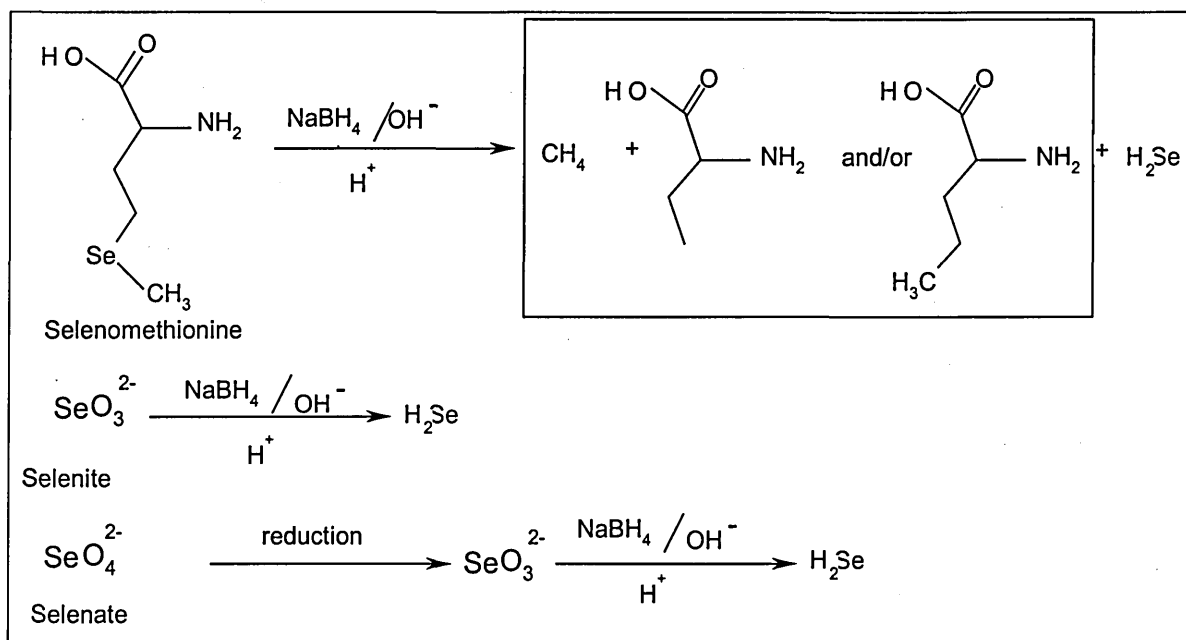


Figure 6. 7 Proposed mechanisms for the formation selenium hydride from selenomethionine, selenate and selenite

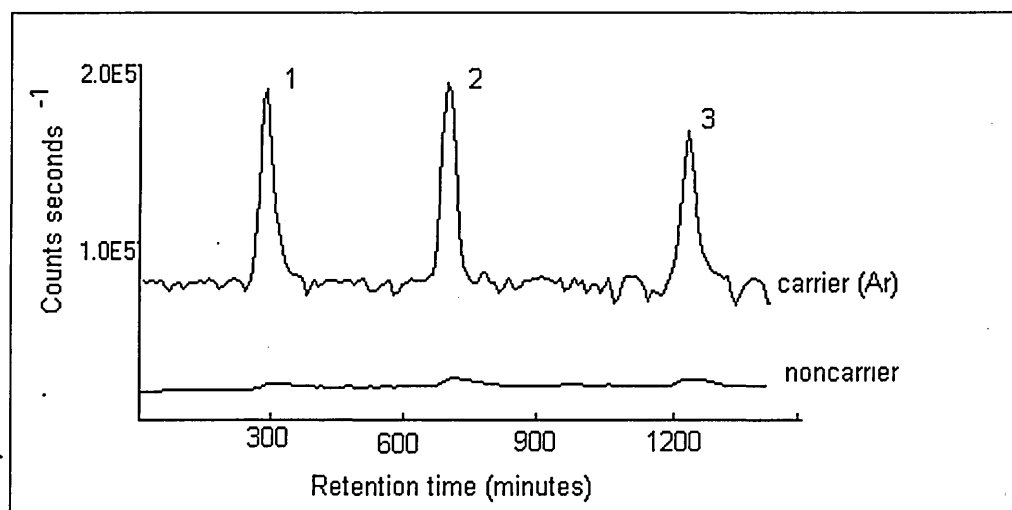


Figure 6. 8 The effect of carrier gas flow on the selenium hydride signal. Dionex ion-exchange AS9-SC 4 mm column, mobile phase: $\text{Na}_2\text{CO}_3/\text{NaHCO}_3$ 1.8/1.7 (0.7 mL min^{-1}); NaOH (0.6 mL min^{-1}), 12 M HCl and 1% NaBH_4 in 0.1 M NaOH (0.8 mL min^{-1}).

Previous research done with the same instrument (78) showed that the optimum carrier flow rate was in the range of $1.25\text{-}1.50 \text{ L min}^{-1}$. As a result the optimum flow rate was fixed at 1.30 L min^{-1} in order to prevent plasma instability.

Table 6. 1 The effect of increasing concentrations of HCl, HBr and NaOH on the photoreduction reaction and formation of selenium hydride

Selenium Species	[HCl]	[HBr]	UV	Photoreduction [■]	
				[NaOH]	[HCl]
Selenomethionine (-II)	0.0 **	6 M *	2.5 M	0.0 **	
	2.0M *			6.0 M **	
	6.0M *			8.4 M *	
	8.4M *			0.0 **	
	11.9M ▲			4.0 M *	
		5.0 M		6.0 M *	
				8.4 M *	
				0.0 **	
				4.0 M *	
				6.0 M *	
Se (IV)	0.0 **	6 M *	2.5 M	0.0 **	
	2.0M *			6.0 M *	
	6.0M *			8.4 M *	
	8.4M *				
	11.9M *			0.0 **	
		5.0 M		4.0 M *	
				6.0 M *	
				8.4 M *	
				0.0 **	
				4.0 M *	
Se (VI)	0.0 **	6 M **	2.5 M	0.0 **	
	2.0M **			6.0 M ▲	
	6.0M **				
	8.4M **				
	11.9M ▲				
		5.0 M		0.0 **	
				4.0 M *	
				6.0 M *	
				8.4 M *	

1.0 % NaBH₄ in 0.1 M NaOH, [■] temperature 80 °C (water bath), * product formed, ** no product obtained, ▲ low yield.

6.2.3 Separation condition

Figure 6. 6.c. shows the chromatogram obtained for the separation of selenomethionine, selenite and selenate on a Dionex AS9-SC ion-exchange column using as the mobile phase a solution of NaHCO₃/Na₂CO₃ with a total eluent concentration of ([HCO₃⁻] + [CO₃²⁻]) = 3.5 mM, and a ratio of [HCO₃⁻]/[CO₃²⁻] 1.8/1.7, at pH 10.2 followed by pre-treatment with 5 M NaOH in the UV-photoreactor and

hydride generation with 8.4 M HCl and 1 % NaBH₄ in 0.1 M NaOH before detection. As shown, good baseline resolutions between the three peaks were obtained. Furthermore, the results show that no deterioration in peak resolution has occurred despite the large dead volume (1.9 mL) in the photoreactor. The α factor and R are summarised in Table 6. 2

Table 6. 2 Chromatographic parameters for the generation of hydrogen selenide (H₂Se).

Parameter	SeMet/selenite	selenite/selenate
α	2.4	1.7
Resolution	3.3	3.8

6.2.4 Calibration graph

Using the optimised conditions, a solution containing 0.2 $\mu\text{g L}^{-1}$ of all three species were separated, and detected. The measurement precision at this level was 18 %. As shown in Figure 6. 10, the signals could be clearly distinguished from the background but quantification was very poor. However, when 0.5 $\mu\text{g L}^{-1}$ was injected, better precision, about 6 %, was obtained. This concentration level was chosen as the limit of determination.

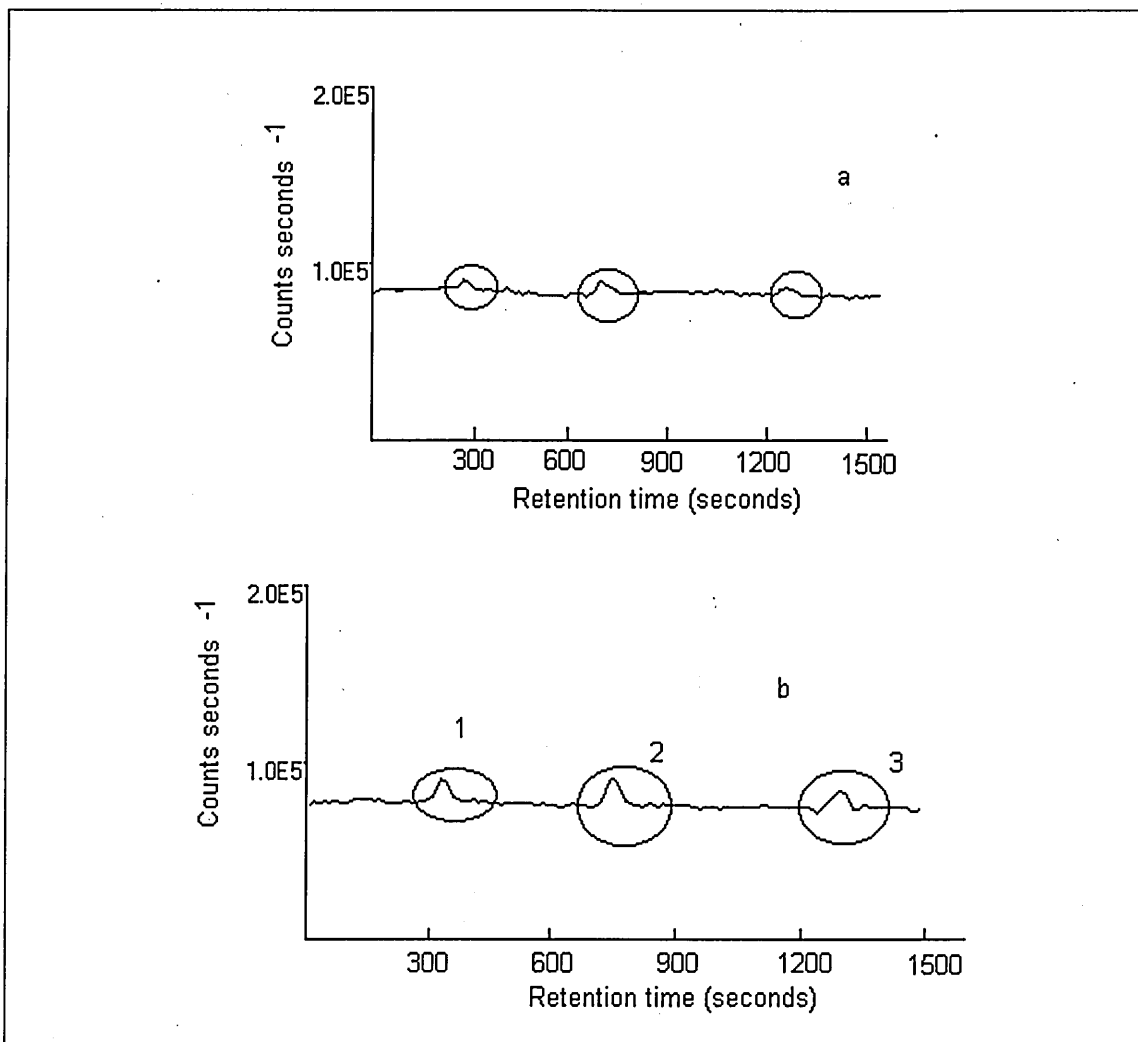


Figure 6. 9 Comparison of the signals obtained from solutions 0.2 and 0.5 $\mu\text{g L}^{-1}$ containing the same concentrations of the three selenium species, selenomethionine (1), selenite (2), selenate (3). Using a Dionex ion-exchange AS9-SC 4 mm column, mobile phase $\text{Na}_2\text{CO}_3/\text{NaHCO}_3$ 1.8/1.7 (0.7 mL min^{-1}); 5 M NaOH (0.6 mL min^{-1}), 8.4 M HCl and 1% NaBH_4 in 0.1 M NaOH (0.8 mL min^{-1}).

A linear calibration graph was obtained between $0.50 \mu\text{g L}^{-1}$ - $200 \mu\text{g L}^{-1}$. The sensitivity obtained with this method is twice that for the solution ICP-MS method (see chapter 4, section 4.2.7), Figure 6. 11 shows the calibration graph for each selenium species. For the method validation a TMDA 23.2 reference solution containing only selenite was analysed, and the selenium concentration found was $3.7 \pm 0.9 \mu\text{g L}^{-1}$, which is close to the expected value of 4.2 ± 1.4 .

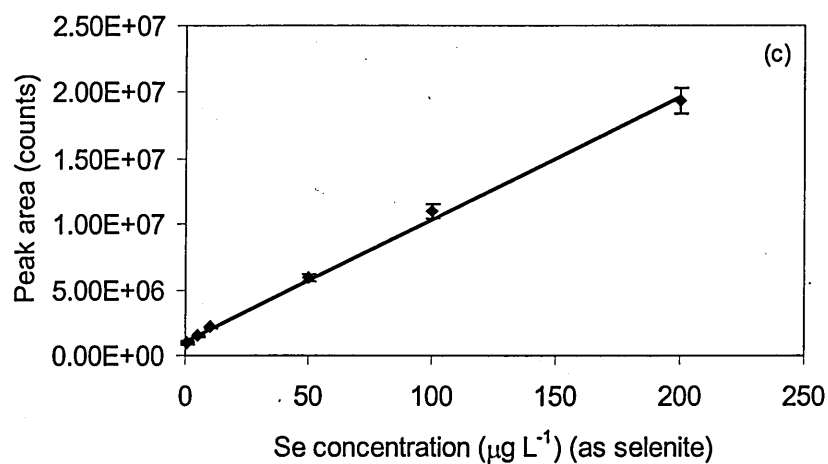
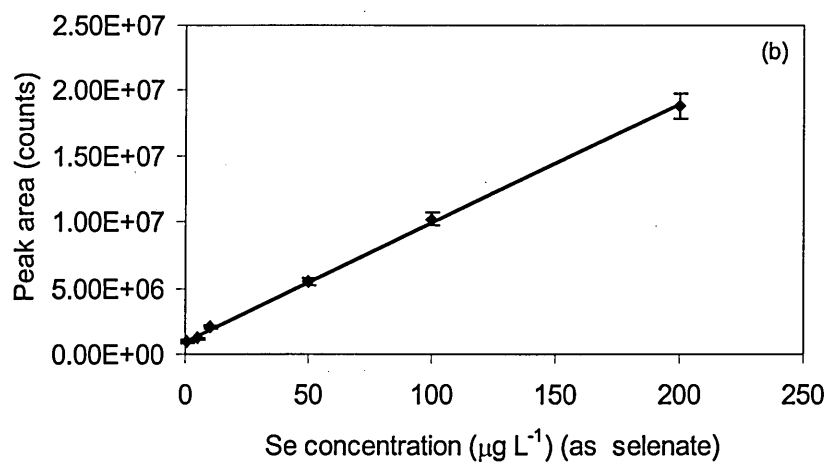
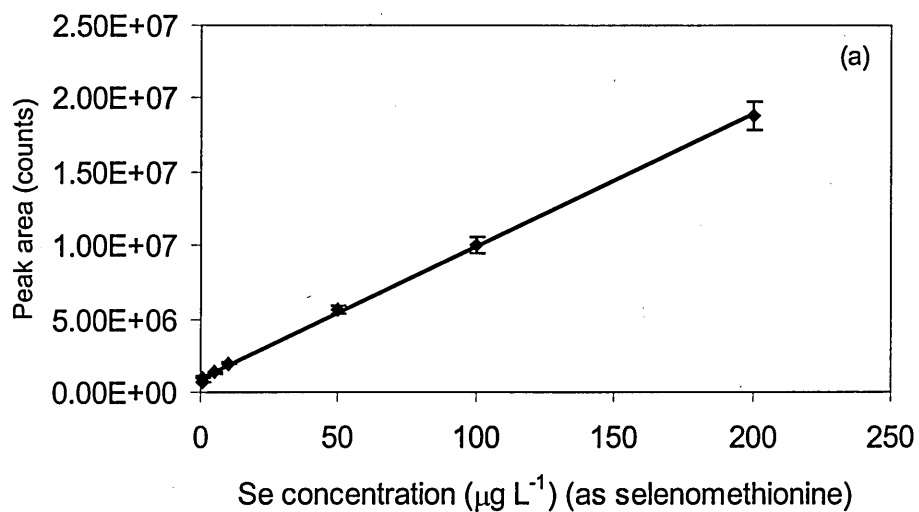


Figure 6. 10 Calibration graphs (a) selenomethionine $y = 90141x + 920595$ $r^2 = 0.9995$, (b) selenite $y = 92799x + 1E+06$ $r^2 = 0.9978$, (c) selenate $y = 89879x + 963116$ $r^2 = 0.9993$.

6.2.5 Sample analysis

Since the samples examined were all solid samples, it was essential to first extract the selenium species without changing their chemical form. One of the extractant used for this purpose is methanol (54). Ideally, it should be possible to inject the extract directly into the column. The behaviour of the three selenium species dissolved in a 1:1 methanol: mobile phase mixture is shown in Figure 6. 11. The presence of methanol caused peak distortions. As a result of the poor separation, and peak distortions associated with the use of methanol, no extraction was carried out in this medium.

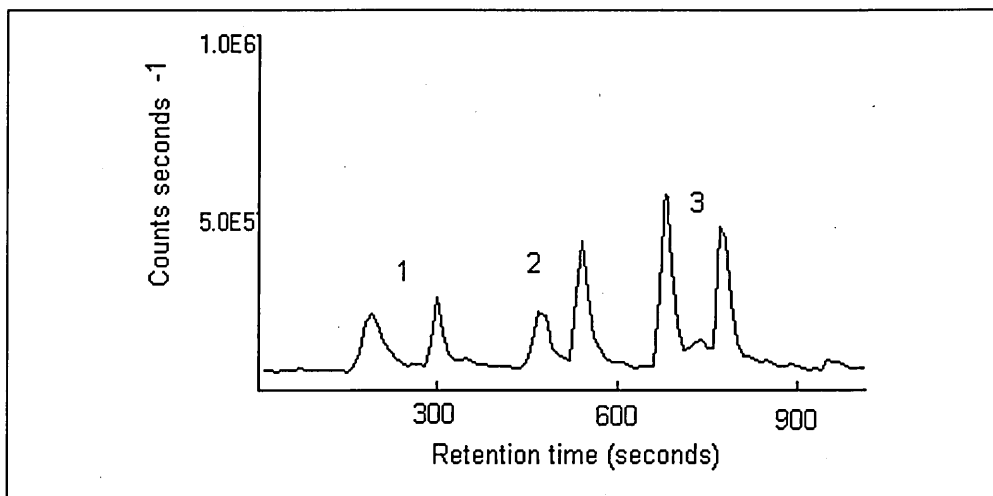


Figure 6. 11 Selenium mixture prepared in methanol/water. Dionex ion-exchange AS9-SC 4 mm column, mobile phase $\text{Na}_2\text{CO}_3/\text{NaHCO}_3$ 1.8/1.7 (0.7 mL min^{-1}); 5 M NaOH (0.6 mL min^{-1}), 8.4 M HCl and 1% NaBH_4 in 0.1 M NaOH (0.8 mL min^{-1}).

For sample A, one peak at 280 seconds was observed corresponding to the retention time for organic selenium (see Figure 6. 12). Similar recoveries were obtained using 2M NaOH, mobile phase or methanol-water.

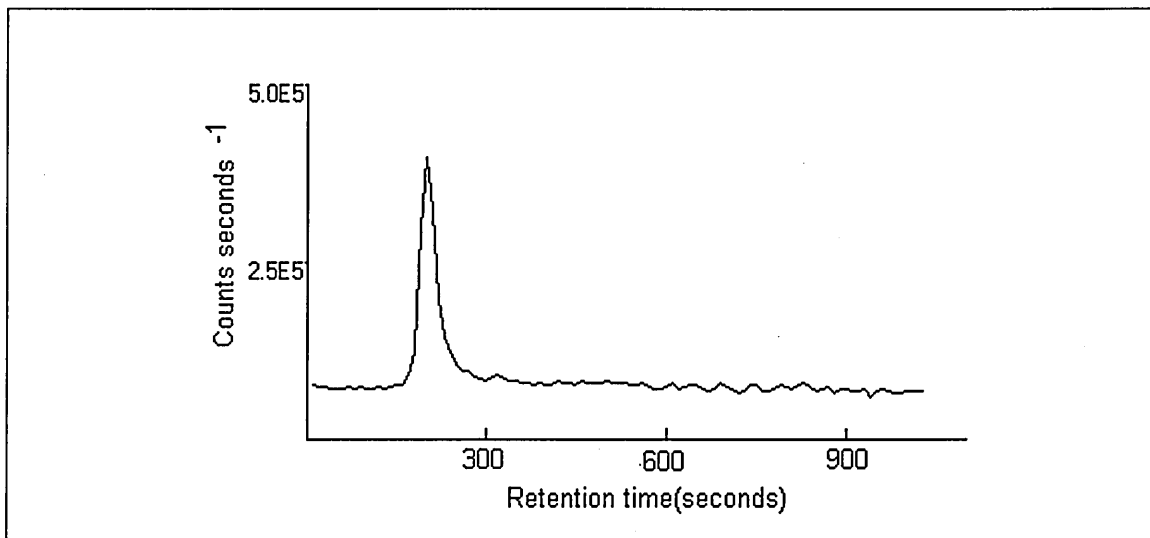


Figure 6. 12 NaOH extract of Sample A. Dionex ion-exchange AS9-SC 4 mm column, mobile phase $\text{Na}_2\text{CO}_3/\text{NaHCO}_3$ 1.8/1.7 (0.7 mL min^{-1}); 5 M NaOH (0.6 mL min^{-1}), 8.4 M HCl and 1% NaBH_4 in 0.1 M NaOH (0.8 mL min^{-1}).

The chromatograph of the sample B, Figure 6. 13, shows that this sample contains selenomethionine and selenite. When the mobile phase was used as an extracting solvent the recovery of Se (IV) was lower than with water. The different observations are shown in Figure 6. 13.b.

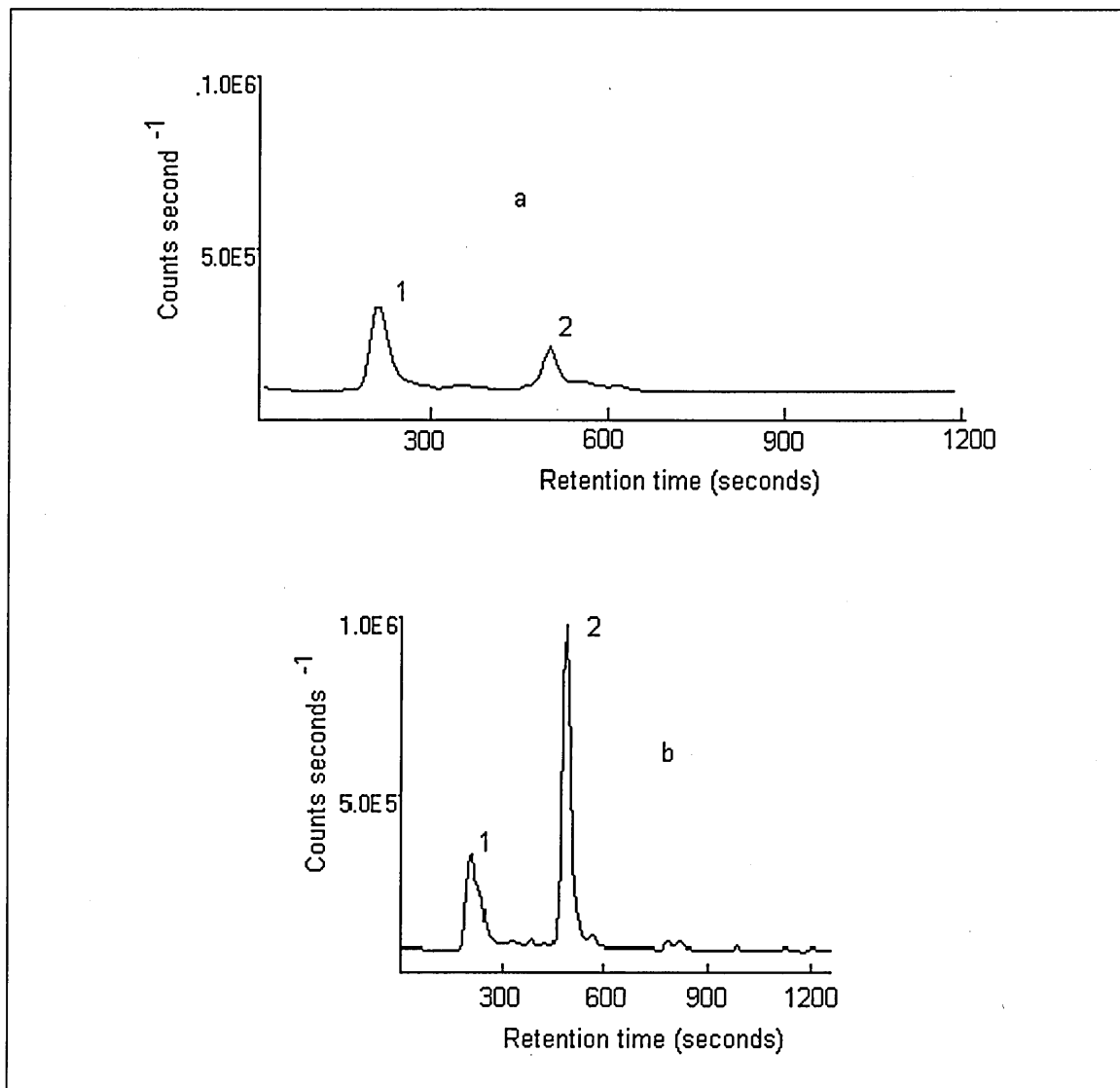


Figure 6. 13 Sample B Analysis. a: mobile phase extract, b: water extract. Dionex ion-exchange AS9-SC 4 mm column, mobile phase $\text{Na}_2\text{CO}_3/\text{NaHCO}_3$ 1.8/1.7 (0.7 mL min^{-1}); NaOH (0.6 mL min^{-1}), 8.4 M HCl and 1% NaBH_4 in 0.1 M NaOH (0.8 mL min^{-1}).

Sample C, which is an oil capsule containing selenium, with vitamins A and E, gave low recoveries of selenium using extraction with water and the mobile phase, whereas using NaOH solution all of the selenium was completely extracted, as seen in Table 6. 3. The higher extraction using NaOH solution is due to the basic medium emulsifying the organic phase of the sample, allowing the selenium species to be liberated into the solution. Selenomethionine was identified as the selenium species present in the sample C from the value of the retention time.

In general, better recoveries were obtained when a mechanical shaker was used for extraction. A comparison of the results obtained with the different methods of selenium extraction is shown in Table 6. 3.

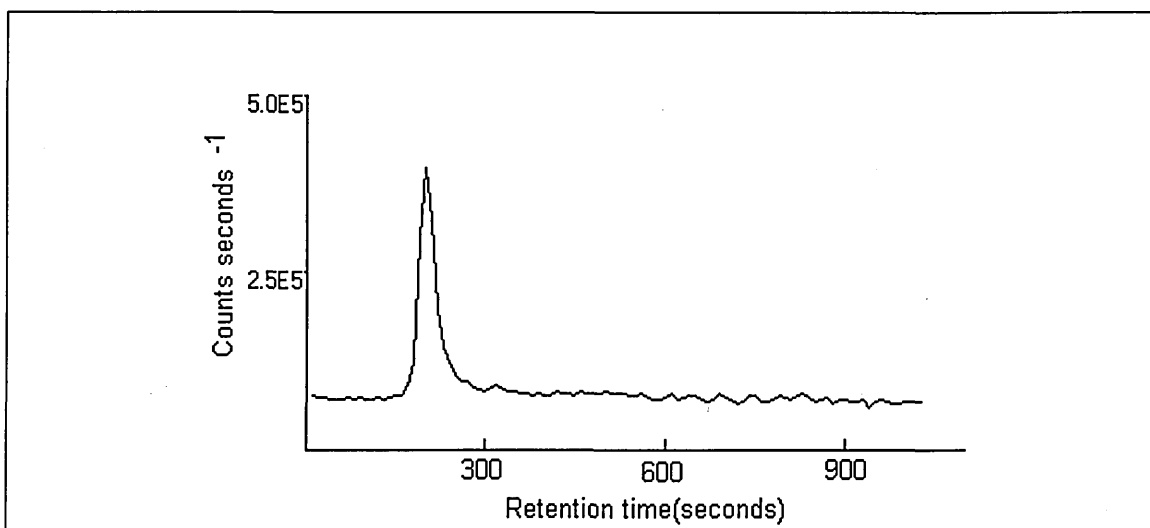


Figure 6. 14 Chromatogram of sample C after extraction with NaOH and separation on an ion-exchange AS9-SC 4 mm, Dionex, mobile phase $\text{Na}_2\text{CO}_3/\text{NaHCO}_3$ 1.8/1.7 (0.7 mL min^{-1}); 5 M NaOH (0.6 mL min^{-1}), 8.4 M HCl and $1\% \text{ NaBH}_4$ in 0.1 M NaOH (0.8 mL min^{-1}).

Table 6. 3 Recoveries of selenium supplements tablets using HPLC-UV-HG-ICP-MS.

		Selenium		Content		$(\mu\text{g L}^{-1})$			
Water		Methanol/ water		Mobile Phase		2.5 M NaOH			
S	Shaker	Ultrason.	Shaker	Ultrason.	Shaker	Ultrason.	Shaker	Ultrason.	
A	*192.7 ± 3.1 (96%)	*165.9 ± 5.5 (83%)	*168.9 ± 9.3 (85%)	*106.3 ± 14.4 (53%)	*183.6 ± 4.5 5 (92%)	*166.8 ± 4.1 (83%)	*197.3 ± 3.7 (99%)	*190.9 ± 3.8 (95%)	
B	*10.4 ± 0.3 $\triangleright 15.3 \pm 0.6$ (62%)	*10.3 ± 0.5 $\triangleright 12.6 \pm 1.1$ (92%)	ND	ND	*9.8 ± 0.4 $\triangleright 4.5 \pm 0.7$ (57%)	*8.4 ± 1.0 $\triangleright \text{ND}$ (33%)	*10.5 ± 0.4 $\triangleright 14.6 \pm 0.5$ (100%)	*10.1 ± 0.2 $\triangleright 13.9 \pm 0.6$ (96%)	
C	*15.5 ± 0.8 (62%)	*14.6 ± 0.9 (62%)	*14.3 ± 1.8 (57%)	*13.8 ± 2.8 (54%)	*18.3 ± 1.1 (73%)	*16.5 ± 0.9 (66%)	*24.8 ± 0.8 (99%)	*23.5 ± 0.9 (94%)	

S = Sample, * selenomethionine, \triangleright selenite.

Table 6. 4 Comparison of selenium determination, in three nutritional supplements, using batch fluorescence, ICP-MS, on-line fluorescence and HPLC-UV-HG-ICP-MS methods.

Sample	Batch Fluorescence $\mu\text{g L}^{-1}$	ICP-MS $\mu\text{g L}^{-1}$	On-line Fluorescence $\mu\text{g L}^{-1}$	HPLC-UV-HG- ICP-MS $\mu\text{g L}^{-1}$
A	200.8 ± 1.6	199.6 ± 9.8	128.6 ± 7.8 *	197.3 ± 3.7
B	25.3 ± 1.1	26.1 ± 1.3	17.9 ± 2.2 *	25.1 ± 0.9
C	25.6 ± 0.9	25.9 ± 1.8	24.8 ± 1.0	24.8 ± 0.8
DL	0.5	1.0	40 Se (VI), 0.5 Se (IV)	0.5 each specie

* Extraction problems.

6.3 CONCLUSION

The use of ion-chromatography with aqueous $\text{Na}_2\text{CO}_3/\text{NaHCO}_3$ as the mobile phase gave a good separation for selenomethionine, selenite and selenate. However the reduction of the selenate to selenite in aqueous HCl or HBr after the separation was not efficient at all. On the other hand, UV-irradiation in a basic medium (5 M NaOH), with post addition of 8.4 M HCl and reaction with NaBH_4 (1% in 0.1 M NaOH) gave a very selective and reliable method for the speciation and quantification of Se (IV), Se (VI) and selenomethionine. The hybrid system linking with ion-HPLC-UV-HG-ICP-MS was easy to use. The developed method has a linear range from 0.5 to 200 $\mu\text{g mL}^{-1}$ of Se. Shaking the sample for extraction for 12 hours with water gave excellent recoveries for samples A and B, but for sample C, it was necessary to add NaOH or HNO_3 to emulsify the organic phase.

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Chapter 7:

General Conclusions and Future Work

7.1 CONCLUSIONS AND FUTURE WORK

Of the four synthetic methods for PTQA investigated, the simplest and most efficient was the condensation of the methyl group of quinaldine with 2-aminopyridine in the presence of elemental sulphur, using sodium sulphide to catalyse the reaction. Molar proportions of 0.10: 0.20: 0.15: 0.08 of quinaldine, 2-aminopyridine, sulphur and sodium sulphide, respectively, at 155 °C for 12 hours, prior to purification on a silica column, gave a product yield of 38.9 %. To improve the PTQA yield, the reaction could be carried out under N₂ atmosphere to avoid the formation of the oxygen analogue of PTQA.

Improved fluorescence sensitivity was obtained in HNO₃ solution, and using a 5 x 10⁻⁴ M PTQA solution in isopropanol. Linear calibration was obtained in the concentration range 40-200 µg L⁻¹. Strong Cl⁻ interference from HCl, used in the reduction of Se (VI) to Se (IV), was avoided by using UV photoreduction in 5 M NaOH. Kinetic studies could be done in order to investigate the mechanism of Se (VI) photoreduction in NaOH.

Application of the developed method to the determination of selenium in nutritional supplements after digestion in H₂O₂/HNO₃ gave excellent recoveries (98.6 %). Results for the analysis of a reference material and nutritional supplements obtained by batch fluorescence method were compared to those from ICP-MS measurements in order to validate the developed analytical procedure. Very good agreement between the two sets of results (r= 0.998) was obtained.

Method automation resulted in shorter analysis time, 8 samples/hr for Se (IV). Simultaneous determination of Se (IV) was possible when a by-pass valve and stopped flow was used to allow the photoreduction of Se (VI) to Se (IV) to occur. The use of an on-line UV photoreactor in which a basic Se (VI) solution was heated at 80 °C for 40 minutes enabled effective reduction and allowed chloride interference free detection. Using this arrangement, it was possible to determine Se (IV) levels in six samples in the same time that one Se (VI) sample was being reduced. As the fluorescence method has shown a dependency on acid concentration, the highest useable HNO₃ concentration should be explored in order to increase the sensitivity of the on-line method.

As a further instrumental development, three simultaneous coils could be attached to the UV-lamp in order to increase the number of Se (VI) samples irradiated.

Excellent separation of selenomethionine, selenite and selenate was achieved using ion chromatography with aqueous $\text{Na}_2\text{CO}_3/\text{NaHCO}_3$ mobile phase on a Dionex ion-exchange AS9-SC 4 mm column coupled with on-line UV photoreduction prior to hydride generation and ICP-MS detection. The time of analysis was 18 minutes per sample. The limit of detection using this hybrid system was $0.50 \mu\text{g L}^{-1}$ for the three species. The developed method was successfully applied to the study of selenium speciation in selenium-containing nutritional supplements.

In future work, either aqueous HCl or HBr could be used for the UV photoreduction of selenate in the HPLC-HG-ICP-MS method in order to avoid dilution caused by medium changes, thus improving the limit of detection.

The methods developed could be applied to the determination of total selenium and used for the study of selenium distribution in environmental, biological and medical samples in order to obtain a better understanding of the element in human health and disease.

These investigations have shown that fluorescence detection provides a cheaper and robust alternative to the more expensive ICP-MS techniques. However, interference studies need to be explored in order to determine possible interference in the fluorescence method, since this is not based on a specific reaction of selenium with PTQA, but is an oxidation reaction catalysed by Se (IV).

Coupling of HPLC-fluorescence detection could also be studied in order to obtain a cheaper and robust method for selenium speciation.